UNIVERSITY OF ALBERTA

TAXOL: SOLUBILITY, STABILITY, AND BIOAVAILABILITY

By

HASHEM MONTASERI ©

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DEDICATED

to my

Father: Late Abbas Montaseri

Mother: Late Ashraf Ghatee

Wife: Batool-Faegheh Zarandi

&

to my

Lovely sons, Alireza and Sina

ABSTRACT

Taxol is one of the more intriguing new anticancer drugs to enter the clinical arena in many years. However, problems including a limited supply of this natural product and its formulation- and dose-dependent side effects (hypersensitivity reactions), have prevented taxol from advancing into the clinical trials.

The oral route may be an alternative to the iv route for administration of taxol in order to reduce the side effects and increase its efficacy. Oral bioavailability of taxol in Sprague-Dawley rat was shown to be very poor (0.16%). Therefore, the objective of this project was to evaluate the oral delivery of taxol and to improve its bioavailability if possible. Studies were performed to identify the physicochemical parameters responsible for poor oral bioavailability of taxol, and different approaches were utilized to overcome this problem. Preformulation studies of taxol, including pH-solubility profile, pH-stability profile, dissolution study and *in vitro* intestinal absorption were conducted using phase solubility experiment, accelerated stability test, dispersed amount method, and everted rat intestine technique, respectively. It was found that low aqueous solubility (0.92 μg/ml at 37^0 C), limited dissolution rate (1 μg/hr at 37^0 C), chemical instability ($t_{1/2}$ 41 and 45 hr at pH 1.2 and 8), and limited gut absorption (< 2%) may account for the low oral bioavailability of taxol. Therefore, Increase in oral bioavailability was found to be feasible by improving solubility and permeability of taxol.

Cyclodextrins, bile salts, nicotinamide, polyethylene glycol copolymers and liposomes were established as suitable solubilizing, stabilizing, and/or permeation enhancer systems capable of enhancing solubility, dissolution, stability, and/or intestinal

permeability of taxol. HPßCD and nicotinamide increased solubility ([illegible] and 180-fold), relative dissolution rate (22- and 42-fold), stability (18.5-fold and -1.7-fold), *in vitro* absorption (2.7- and 4-fold), and oral bioavailability (6- and 15-fold) of taxol, respectively. Results of IAM column chromatography and intestinal permeability (^{51}Cr-EDTA) studies showed that HPßCD or nicotinamide increased oral bioavailability of taxol mostly through a change in permeability of the tight junctions in the endothelium of gut wall. Therefore, by using GI permeability enhancing effect of NSAIDs (flurbiprofen, 10 mg/kg, 0.5 hour before administration of taxol), the oral bioavailability of taxol was increased to 23%.

The results highlighted the rule of poor solubility and permeability of taxol in its overall low bioavailability and the potential application of HPβCD, nicotinamide, and NSAIDs as enhancers of solubility, permeability, and bioavailability of taxol.

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GLOSSARY OF ABREVIATIONS

αCD	alpha-cyclodextrin
ANOVA	analysis of variance
AUC	area under the plasma concentration-time curve
ß	elimination rate constant
ßCD	beta-cyclodextrin
BS	bile salts
CHOL	cholesterol
CL/F	oral clearance
CL_T	total clearance (systemic clearance)
C_{max}	peak concentration
CR	cremophor EL
DPPC	dipalmitoylphosphatidylcholine
DSC	differential scanning calorimetry
F	absolute bioavailability
FB	flurbiprofen
γCD	gamma-cyclodextrin
g	gram
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HPßCD	hydroxypropyl-beta-cyclodextrin
hr	hour
IAM	immobilized artificial membrane
i.d.	inner diameter
ip	intraperitoneal
iv	intravenous
Kg	kilogram
L	liter
µg	microgram

µl	microliter
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mmol	millimole
n	number of observation
ND	nicotinamide
ng	nanogram
NSAID	nonsteroidal anti-inflammatory drug
°C	degree Celsius
o.d.	outer diameter
PEG	polyethyleneglycol
PEG-PBLA	poly(ethyleneglycol)-poly(beta-benzyl-L-aspartate)
pH	negative logarithm (base 10) of the hydrogen ion concentration
po	per oral
rpm	revolutions per minute
SEM	standard error of the mean
STDEV	standard deviation
$t_{1/2}$	half-life
TD_{50}	dose required to kill 50% of cells
T_{max}	time to maximal plasma concentration
UV	ultraviolet
V_d	volume of distribution
V_{dss}	volume of distribution at steady state

Chapter 1

1. Introduction

1.1 Taxol

1.2 History

Taxol is a prototype of taxane containing natural products which exhibit antineoplastic activity. Although the scientific history of taxol started over thirty four years ago, a clarified-butter preparation containing European yew (*Taxus bacata*) had been used in traditional medicine of India long before that time (Hoemel 1982). Taxol entered in the large scale cytotoxicity screening program of the National Cancer Institute of the United States (NCI) in 1962 (Gregory and Delisa 1993), and was first isolated from the bark of Western yew (*Taxus brevifolia*) in 1967. Even though the detailed structure and the *in vitro* cytotoxic activity of taxol were reported by Wani *et al.* in 1971, it was until the late 1970s that scientists at NCI showed interest in this compound. The mechanism of action of taxol was studied in the late 1970s and found to be unique among plant alkaloids and other anticancer drugs (Rowinsky *et al.* 1992). Preclinical studies in experimental animals confirmed the activity of taxol against several tumors. Phase I trials of taxol were conducted in the early 1980s (Leaha *et al.* 1986, Grem *et al.* 1986, Kris *et al.* 1986, Donehower *et al.* 1987, Rowinsky *et al.* 1989, Wiernik *et al.* 1987a, 1987b). Despite the promising antitumor activity against a variety of tumors that were observed in those trials, further development of this drug was delayed due to its insolubility in water, insensitivity of available analytical methods, and hypersensitivity reactions (Koeller and Dorr, 1994). However, these problems were conquered by formulating taxol in cremophor EL, developing some biochemical assay, and administering antihypersensitivity agents prior to each taxol dose.

By the mid-1980s the first Phase II trials of taxol as a single agent was started in patients with tumors that responded to the drug in the Phase I trials. In all of these trials patients were pretreated to prevent hypersensitivity reactions. Several tumors were highly responsive to taxol with objective response rates higher than 50 percent for breast cancer, 30 percent or higher for ovarian cancer, and higher than 20 percent for non-small-cell lung cancer (NSCLC). These response rates were equal to, or better than the rates achieved with standard primary or salvage chemotherapy. More importantly, many of the patients in these studies were heavily pretreated and had failed prior therapy (Rowinsky 1992). Since 1992 taxol has been approved in the US for treatment of patients with advanced ovarian cancer whose prior platinum-containing standard therapy failed. Clinical results from completed Phase II trials in ovarian, breast, and non-small-cell lung cancers suggest the potential of taxol as a wide spectrum antineoplastic agent. Based on the evidence of taxol's safety and efficacy for treatment of refractory ovarian cancer NCI, in an open competition, selected Bristol-Myers Squibb Company as its pharmaceutical partner to obtain approval for New Drug Application (NDA). At present, taxol under the name of Paclitaxel®, is marketed by Bristol-Myers Squibb as single dose 5 ml vials containing 30 mg of taxol in cremophor EL. Ongoing and future trials are designed to explore taxol in combination with other effective agents or as primary chemotherapy, the management of significant toxicities, pharmacodynamics and pharmacokinetics, with emphasize on its formulation and route of administration.

1.2.1 Physicochemical Properties

The chemical structure of taxol ($C_{46}H_{49}O_{14}N$) or [benzenepropanoic acid, beta-(benzoylamino)-alpha-hydroxy-6,12b-bis(acetyloxy)-12-(benzoyloxy)-2a,3,4,4a,5,6, 9,10,11,12,12a, 12b-dodecahydro-4,11-dihydroxy-4a,8,13,13-tetramethyl-5-oxo-7,11-methano-1H-cyclodeca(3,4)benz(1,2-b)oxet-9-yl ester, (2aR-(2a-alpha,4beta,4a-beta,-6beta,9alpha(alphaR*,betaS*),11alpha,1-2alpha,12a-alpha, 12b-alpha)-(9CI)], is shown in figure 1-1-a. Taxol is chemically categorised as a complex diterpenoid with a taxane nucleus and a phenylisoserine side chain at C-13 of its molecule. Although taxol shares some structural similarities with other natural alkaloids such as vincristine and vinblastine, its taxane nucleus is unique (Figures 1-1-b and 1-1-c).

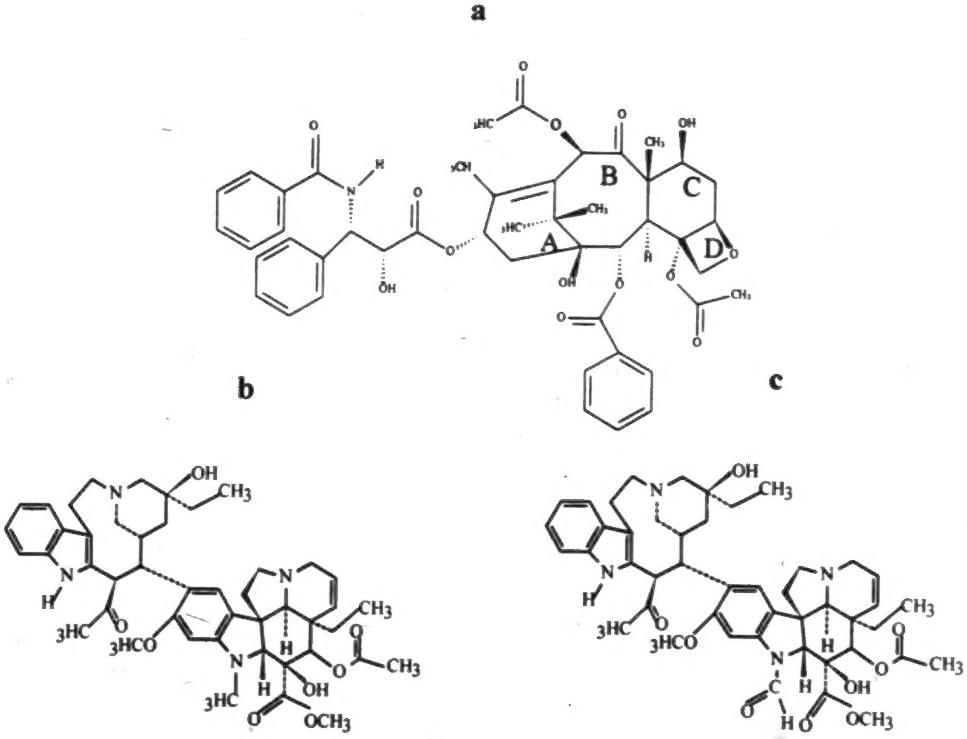

Figure 1-1 Structures of a) taxol, b) vinblastine, and c) vincristine

The taxane nucleus consists of four rings, A (6-membered system), B (8-membered system), C (6-membered system), and D (Oxatane ring). From the physicochemical point of view the taxol structure can be divided into two sections, a polar and a nonpolar section. These two distinct sections are quite important in the intraction of taxol with its receptor (Figure 1-2).

The commercially available taxol is a white, odourless, amorphous, hygroscopic powder with a purity of 98% (by HPLC) and molecular weight of 853.9. Taxol is practically insoluble in water and has a maximum UV absorption at 227 nm, melting point of 205-208° C, and octanol/buffer partitioning of more than 99.

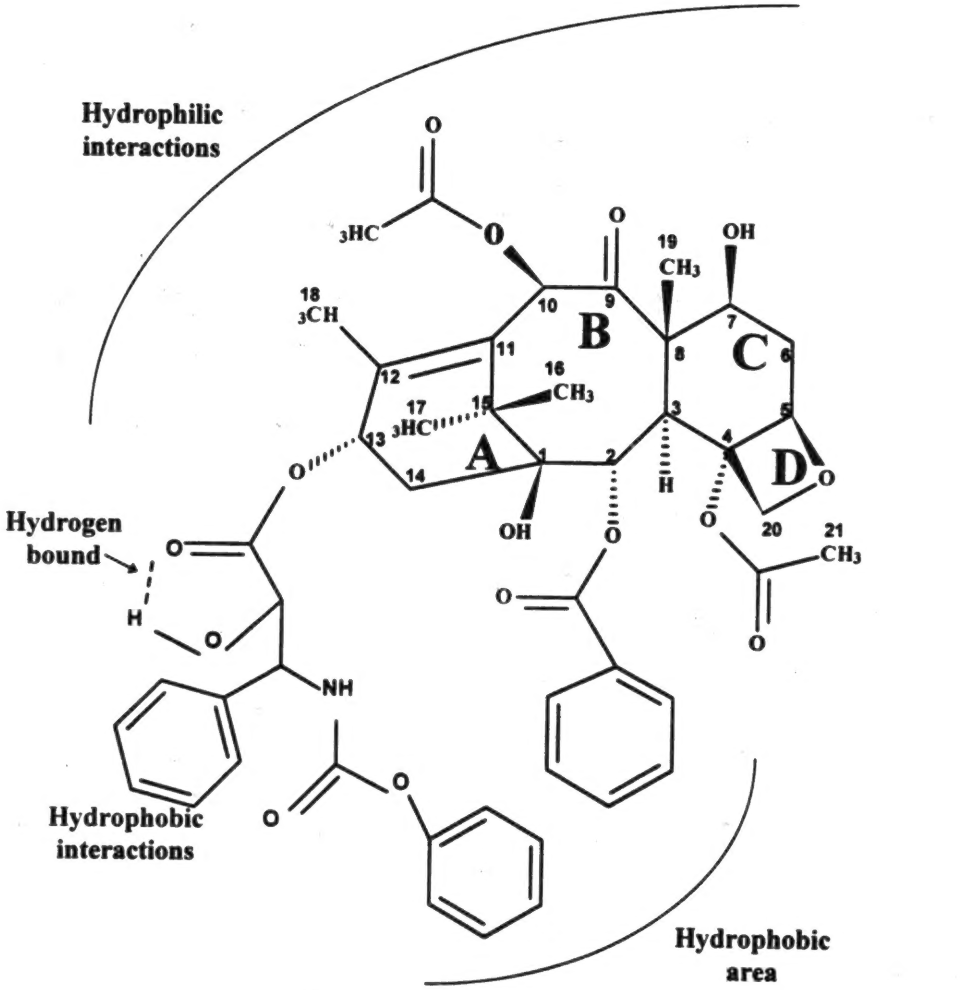
Hydrophilic interactions
Hydrogen bound
Hydrophobic interactions
Hydrophobic area
A
B
C
D
OH
H
NH
O
3HC
18
3CH
16
CH3
17
3HC
19
CH3
21
CH3
20
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 1-2 Configuration of different functional groups in the structure of taxol

1.2.2 Source

Taxol was first isolated in 1967 from the bark of taxus brevifolia (Western yew or Pacific yew). Western yew is a shrub, 20-40 feet in height, and 12-15 inches diameter and a life span of 250-350 years. Yew regenerates by its seed, stump, and root sprouts. The amount of taxol in different parts of Western yew varies between 0.00002 to 0.057% but dried bark contains the greatest concentration of taxol (0.01-0.057%). The amount of taxol in Taxus brevifolia is so low that at least three 100 year old trees are necessary to treat one patient. Different groups are continuing to work on the production of taxol ranging from biomass collecting from wild spices, cell culture, semisynthesis and total synthesis (Vance and Kelsey 1992, Koepp *et al.*, 1995).

1.2.2.1 Biomass Collection

The Pacific yew has recently gained significant attention as the principal source of taxol. Stull *et al.* in 1992 reported that about 130 million yew trees of all sizes are growing on land, but only 23 million of them are suitable for harvesting. In 1992 taxol production technology yielded 1 kilogram of taxol from 16000 pounds of dried bark (2000-3000 yew trees). The production capacity in 1992 was about 130 kilograms per year, but since then the production has been expanded to more than 230 kg per year.

1.2.2.2 Tissue Culture

Taxol production by fermentation technique, using an endophytic fungus of Pacific yew (Taxomyces andreanae), and plant cell culture or tissue culture methods have the potential to provide sufficient quantities of taxol for chemotherapeutic use (Stierle *et al.*, 1993, and Shuler *et al.* 1992). Cell culture offers the potential of a reliable and renewable resource of material, while current bark stripping methods lead to the destruction of the existing plants. Tissue culture process is also more adaptable to increases in demand for taxol. The highest yield in suspension culture has reported as 3.9 mg/L taxol in the median after 26 days of culture (Shuler *et al.* 1992). However, these biotechnological methods are labour intensive and required specialized facilities.

1.2.2.3 Semi-synthesis

A practical solution to the taxol supply problem is now available through semi-synthesis. Retrosynthetic analysis of taxol presents three challenges:

1-Synthesis of the N-benzoyl-(2R, 3S)-3 phenylisoserine side chain.

2-Synthesis or extraction of bacatin III from the needles of yew trees.

3-An effective esterification protocol for coupling of bacatin III and Phenylisoserine

The synthesis of bacatin III is more difficult and lengthy. While 10-deacetylated bacatin III can be extracted from the needles of different yews in a yield of approximately 0.1% (Taxuse bacata) which provides a practical resolution to taxol supply issue through reattachment of taxol side chain (Holton *et al.* 1992). This approach would provide security of supply and quality control, for taxol. (Rao *et al.*, 1995, and Koepp *et al.*, 1995).

1.2.2.4 Synthesis

Synthesis plays a unique role in the advancement of taxol supply. Total synthesis could provide a practical and reliable source of taxol that is not dependent on the biosynthetic processes. Although the synthesis of the whole as well as a partial structure of taxol has been successfully done since 1994 (first reported by Nicolaou *et al.* 1994), the approach is very complex and difficult to scale up. Therefore, with today's technology, the replacement of initial natural source of taxol with totally synthetic taxol does not seem to be commercially feasible.

1.2.3 Pharmacology

1.2.3.1 Mechanism of Action

The mechanism of action of taxol differs from that of other antineoplastic agents, although it affects similar intracellular structures. Taxol targets microtubules, which are polymeric intracellular structures composed of chains of a dimeric protein tubulin.

Microtubules are involved in a diverse variety of cellular processes including segregation of chromosomes during mitosis and miosis, maintenance of cell shape, motility of flagella and ciliary, and transport of organella. Unlike other antimicrotubule agents, such as the vinca alkaloids, colchicine, colcemid, griseofulvin, maytansine, podophyllotoxin, and steganacin which induce the depolymerization of microtubules (disassembly), taxol is known to increase the microtubuline polymerisation (Schiff *et al.*, 1979, 1980, 1981, Kumar 1981, Manfredi *et al.*, 1984). Such increase however, results in abnormal, stable, assembled, and nonfunctional microtubules (clumped).

Schiff *et al.*, and Hamel *et al.*, in 1981 explained that taxol concentrations as low as 0.05 µmol/L decreases the critical concentration of tubulin required for microtubule assembly in the presence or absence of factors, such as exogenous GTP and MAPs, that are usually essential for the assembly. Although the binding site for taxol on microtubules has been shown to be distinct from the binding sites for colchicine, podophyllotoxin, and vinblastine, the exact binding site for taxol on microtubules has not been identified yet. However, it has been demonstrated that taxol binds preferentially to microtubules rather than to tubulin dimers (Parness *et al.*, 1981).

Taxol-treated microtubules are stable even after treatment with calcium or low temperatures, which usually promote disassembly (Thompson *et al.* 1981). This unusual stability inhibits the normal dynamic reorganization of the microtubule network. Taxol concentrations of 0.1-10 µmol/L, which can be clinically achieved with prolonged infusions, produce two distinct morphologic effects on cellular microtubules.

First, in the presence of taxol, cells form abundant arrays of disorganized microtubules aligned in parallel bundles. Since bundles form during all phases of the cell cycle (Rowinsky *et al.*, 1988, Roberts *et al.*, 1989 and 1990), the disruption of the mitotic spindle causes the arrest of cell division in the G2/M phase. Second, taxol causes exceptional stability in microtubules structure, which can depress the effects of essential intracellular regularity elements on this system and thereby alters microtubule dynamics and deprives the cell of its ability to control and organise the cytoskeleton.

Due to these effects on microtubules, taxol appears to block mitotic spindle formation during cell division, as well as the integrity of interphase microtubules that are critical in many cell functions.

1.2.3.2 Structure Activity Features

Taxol has an unusual structure, which accounts for many of its unique pharmacologic and clinical characteristics, as well as its unique mechanism of action (McKay 1989). Taxol is currently being investigated for use both as a cancer chemotherapy agent and as a tool to investigate microtubule involvement with various cellular functions. Many studies have been designed to establish structural requirements for taxol's effects (Gueritte-Voegeleine *et al.* 1991, Samaranayake *et al.* 1991). The results from these studies suggest:

1-Microtubule assembly is quite sensitive to the configuration of the C_2 and C_3 rings.
2-Microtubuline assembly is dependent on the oxatane ring.
3-Estrification of C_{13}-OH is essential for activation of taxol.
4-Replacment of $3'$ phenyl with a methyl group resulted in a major loss of activity.
5-Esterification of the C_2-OH and C_7-OH increase the of solubility of taxol but decreases microtubule assembly, *in vitro*.
6-The presence of a phenyl group at $C_{3'}$ and a hydroxyl group at $C_{2'}$ has little effect on the side chain configuration but is essential for taxol interaction with microtubuline.
7-The acetyl group at C_{10} is not necessary for *in vitro* activity.
8-The amide substitution at $C_{3'}$ by many hydrophobic substituents may increase the potency of taxol, (e.g. taxoter).

Taxoter (docetaxel/Rhone-Poulenc Rorer) is a semisynthetic derivatives of taxol. In some studies, it has been found to have greater cytotoxic effects than taxol on human tumor cells (Hanauske *et al.*, 1992). Although taxoter was reported to be effective against advanced breast cancer and certain types of lung cancer, the Food and Drug Administration (FDA) committee stated that further studies of side effects, especially effects on the immune system, are required. Taxoter is apparently more toxic to patients than taxol and still has not been approved by the FDA.

1.2.3.3 Resistance

Two mechanisms of acquired resistance to taxol have been characterized *in vitro* (Rowinsky *et al.* 1992). First, cells may mutate so that they lack normal microtubules in their mitotic spindles when grown in the absence of taxol. These cells become partly or completely dependent on the presence of the drug for normal growth to occur. The second mechanism involves the multidrug resistance (MDR) phenotype, which confers varying degrees of cross-resistance to taxol and other natural products, including the vinca alkaloids and the anthracyclines.

1.2.4 Preclinical Information

1.2.4.1 Antitumor Activity

In 1971 Wani MC *et al.*, reported that taxol showed moderate cytotoxic activity against murine L1210, P388, P1534 leukemias, Walker 256 carcinosarcoma, sarcoma 180, Lewis lung carcinoma, and KB cell lines. It was not selected for clinical development by the NCI until the late 1970s. That was the time when the unique mechanism of action and broad spectrum of activity of taxol both in murine tumors and human xenografts was confirmed by NCI thorough large panel screening. Taxol's most impressive activity was against the relatively resistant murine B16 melanoma. It also demonstrated good, but not particularly impressive antitumor activity against several human tumors xenografts in the NCI screening panel of the late 1970s. This included good activity against the MX-1 mammary tumor implanted beneath the renal capsule of athymic mice when taxol was given subcutaneously (sc), and moderate activity against human CX-1 colon and LX-1 lung xenografts and intraperitoneally (ip) implanted P388 and L1210 murine leukemias. It was ineffective against the murine sc-implanted Lewis lung carcinoma. Taxol also demonstrated good cytotoxic activity against several human leukemias and solid tumors in early studies performed by several independent investigators. For example, Riondel and Jacrot (1986) reported that it induced significant growth delays in human endometrial, ovarian, brain, tongue, and lung tumor xenograft.

Substantial regression in 4 of 5 human breast carcinoma implants in athymic mice also has been noted. In 1988 the same investigators compared the effects of taxol and a congener with different substitution at C_{13} substituent, and showed that taxol was significantly active against a human ovarian xenograft in nude mice, while the congener was inactive. It has also been reported that taxol is inactive against sc implants of human pancreatic carcinoma (Sternberg *et al.*, 1987). In later studies, Rose *et al.* (1992), evaluated the activity of taxol in several murine and human xenograft models. Treatment/control values ranged from moderate (P388: 130-190%) to excellent (M109 and B16: > 250%).

Schedules using prolonged and frequent drug administrations were superior to bolus and less frequent administration. With the exception of the combination of cisplatin and taxol, which produced a significant delay in tumor growth but not ILS over taxol alone, combinations of taxol with VP-16, doxorubicin, cyclophosphamide, methotrexate, pentamethylmelamine, and bleomycin did not significantly affect antitumor activity or ILS.

1.2.4.2 Animal Toxicity

The preclinical toxicology of taxol was evaluated in mice, rats, and dogs. In rodents, the ip route of administration was used during toxicologic and screening studies due to dose-volume constraints imposed by taxol's limited aqueous solubility and the toxicity of the cremophor EL (polyoxyethylated castor oil) and ethanol vehicle formulation. Toxic effects were most evident in tissues with rapid cell turnover such as hematopoietic, lymphatic, gastrointestinal, and reproductive tissues. Hematopoietic toxicity was observed in all three species. In dogs, myelosuppression was cumulative and reversible on both the single and the daily x 5 dosing schedules. Effects on nervous, hepatic, cardiovascular, and renal tissues were minor, with no postmortem evidence of end-organ damage. The vehicle in which taxol was formulated has been shown to have inherent toxicity (Lorenz *et al.* 1977). In toxicologic studies, cremophor EL, given in increasing doses without taxol as a large-volume single dose, produced vasodilatation, labored breathing, lethargy, hypotension, and death in dogs. The vehicle was much better tolerated in the repeated dose regimen and did not have cumulative toxicity. Although dogs were previously reported to be extremely sensitive to the hypotensive effects of the cremophor EL component of the vehicle, these results suggested that clinical studies should be preformed cautiously.

1.2.5 Clinical Information

1.2.5.1 Antitumor Activity in Clinical Trials

Taxol (Paclitaxel®) has been approved by the FDA for use in patients with recurrent ovarian cancer after failure of first-line or subsequent chemotherapy regimens. It has also been approved for the treatment of patients with breast cancer after failure of combination chemotherapy for metastatic conditions or for relapse within 6 months of treatment with adjuvant chemotherapy. According to the NCI taxol has been or is under clinical investigation in the following areas: Phase I (breast cancer, liver metastases/dysfunction, lymphoma, ovarian epithelial cancer, primary liver cancer, primary peritoneal cancer, refractory childhood leukemia, and some other solid tumors), Phase II acute myelogenous leukemia, breast cancer, cervical cancer, colon cancer, endometrial cancer, esophageal cancer, germ cell tumors, head and neck cancer, melanoma, nonsmall cell lung cancer, ovarian epithelial cancer, prostate cancer, renal cell cancer, small cell lung cancer, soft tissue sarcoma, urothelial cancer), and Phase III (ovarian epithelial cancer and recurrent metastatic breast cancer). Results from both completed and ongoing Phase I, II and III studies are shown in Table 1-1.

These results provide evidence that taxol is active as salvage therapy in patients with several advanced or metastatic tumor types, including ovarian, breast, NSCLC, small-cell, and head and neck cancers. The response rates achieved in these tumors are particularly impressive because heavily pretreated patients, and those who are refractory to prior standard chemotherapy, have had a high rate of objective responses to taxol. The activity of taxol in melanoma and in renal cell, prostate, colon, and cervical cancers, however, does not appear to be especially promising at this time. Use of taxol also is being investigated in bladder, esophageal, and stomach cancers, as well as pediatric malignancies, including leukemias and solid tumors.

Table 1-1 Phase I and II studies of clinical activity of taxol

Target tumor	%Response	References
Advance ovarian cancer	36% (20-50%)	Sarosy *et al.* 1992a,b, McGuire *et al.* 1989, Enzig *et al.* 1992
Metastatic breast cancer (with only one prior chemotherapy).	59% (56-62%)	Holmes *et al.* 1992, Seidman *et al.* 1992,
Metastatic breast cancer (without any treatment)	62%	Seidman *et al.* 1992
Untreated Non Small Cell Lung Cancer (NSCLC)	22 % (21-24%)	Murphy *et al.* 1993, Chang *et al.* 1993
Small Cell Lung Cancer (SCLC)	34%	Ettinger *et al.* 1993
Colon	Limited	Rowinsky *et al.* 1992
Prostate	Limited	Roth *et al.* 1992
Renal	No effect	Einzing *et al.* 1991
Malignant melanoma	16% (12-33%)	Wiernik *et al.* 1987, Wiernik *et al.* 1992 Einzing *et al.* 1991
Head and neck	40%	Forastier *et al.* 1994

1.2.5.2 Combination Chemotherapy

Due to the encouraging results of Phase II trials with single-agent taxol in several tumor types, the next logical step in the development of this agent has been to combine it with other active agents in these tumors. The first combination to be studied was taxol and cisplatin and the second was taxol and doxorubicin. Other drug combinations under study are carboplatin/taxol, cyclophosphamide/taxol, etoposide/taxol, hexamethylmelamine/taxol, ifosfamide/taxol, R-verapamil/taxol, carboplatin/cisplatin/taxol, and cisplatin/cyclophosphamide/taxol. The ongoing studies should clearly define the role of taxol in ovarian cancer. The role of this agent in breast cancer will be defined when the optimal use of taxol in combination with other drugs is determined. Future studies in other cancers, including NSCLC and head and neck cancer, will use combinations of cisplatin and taxol. It will be important to determine if there is a dose-response relationship in the range of dosages currently-used clinically. Important unresolved issues in the development of taxol include its role in primary therapy of solid tumors, the optimal length of its infusion, a clearer understanding of the toxicities associated with taxol and the development of methods to prevent or minimize these toxicities, and the efficacy and safety of taxol in combination with other agents.

1.2.5.3 Radiosensitizer Action

Since cells in the G2 and M phases of the cell cycle are particularly sensitive to radiation and because taxol selectively blocks cells in these phases, several studies conducted to determine whether taxol sensitizes cells to radiation. Tishler *et al.* (1992a and 1992b) showed that when a relatively radiation-resistant human, high-grade astrocytoma cell line (G18) was exposed to noncytotoxic concentrations of taxol (10-100 nM/L), these cells became sensitized to the cytotoxic effects of ionizing radiation, with the degree of enhanced killing dependent on the concentration of taxol and the fraction of cells in the G_2 and M phases of the cell cycle. The feasibility of using taxol as a radiosensitizer is currently being evaluated by a number of institutions, primarily in patients with NSCLC, esophageal, breast, and head and neck cancers.

1.2.5.4 Human Toxicity

A series of Phase I trials of taxol began in the early 1980s that delineated the drug's major toxicities. In all of these trials, taxol was administered as a 24-hr continuous intravenous infusion, repeated once every 3 weeks, with dose reductions in subsequent courses based on the occurrence of severe hematologic or neurologic toxicities. Prior to each course of taxol, patients in these trials were pretreated with an antihypersensitivity regimen generally consisting of an antihistamine, an H_1-blocker, and an H_2-blocker.

In most of these trials neutropenia was the dose limiting side effect, with a proportional increase in severe neutropenia as the dosages of taxol were increased from < 150 to > 190 mg/m^2. In trials using repeated doses of >190 mg/m^2, administered on a prolonged 24 hr infusion schedule, neurotoxicity was demonstrated to be the principal toxicity. Other toxicities, including allopecia in all patient, myalgias, mucositis, nausea, vomiting, diarrhea, and hepatic toxicity, also were found to be dose related. Two toxicities that seemed to be independent of dose were hypersensitivity reactions and cardiotoxicity. Hypersensitivity reaction including flushing, rash, dyspnea, hypotension and chest pain appears in the first 10 min of infusion. Pretreatment with dexamethasone, cimetidine, diphenhydramine can decrease the incidence of severe hypersensitivity. As of July 1992 many patients have entered in taxol research programs and to date only five deaths associated with neutropenic sepsis have been reported. Despite these findings of dose-toxicity relationships, it has not been possible to define a dose-response relationship for antitumor activity of taxol.

1.2.5.5 Metabolism

Most of the taxol disposition and clearance are due to one or a combination of tissue binding, metabolism or biliary excretion, but hepatic metabolism and biliary excretion are potentially important mechanisms involved in the disposition of taxol. No metabolites of taxol were identified in plasma or urine in early phase I studies, except for a new peak on reverse phase HPLC that eluated before taxol in plasma of a single patient receiving taxol over 6 hrs (Rizzo *et al.*, 1990). Using a highly sensitive HPLC assay, however, Beijne (1993) has detected several new possible metabolite peaks at the end of infusion in plasma of patients receiving brief 3 hr infusions of taxol.

High concentrations of taxol and hydroxylated metabolites have been identified in both rat and human bile (Monsarrat *et al.*, 1990, 1993; Wright *et al.*, 1992). It has been reported that only 10% of a dose of taxol (10 mg/kg iv) administered to rats could be eliminated by renal excretion; however, no metabolite was detected in rat urine. In humans, 10% of the administered dose of taxol could be detected in urine as the parent drug.

In rats, taxol appears in the bile within 5 minutes after injection and reaches its maximum rate of elimination after 15 minutes, and is still detectable in bile 24 hrs after injection. It has been reported that over 40% of the total dose of the injected taxol can be recovered (11.5% as taxol and 29% as its metabolites) in the bile of Sprague-Dawley rats during the 24 hr period after injection. In humans it has been shown that approximately 20% of the administered dose of taxol, is recovered as both parent compound and metabolites during the first 24 hrs after treatment. Following the administration of ^{14}C labeled taxol, Gaver *et al.*, (1993) have recovered 90% of radioactivity in rat feces (collected for 6 days) whereas they recovered less than 10% of radioactivity in the rat urine.

Detection and identification of both human and rat metabolites in bile were reported using analytical HPLC, mass spectrometry, and nuclear magnetic resonance spectroscopy (Monsarrat *et al.*, 1990, 1992, 1993; Wright *et al.*, 1992). Neither glucuronidated nor sulphated metabolites were identified, however, nine and five metabolites peaks have been detected in rat and human bile, respectively. To date, all metabolite peaks detected in humans have intact side chains at position C_2 and C_{13} of the taxane ring. Baccatin III, which lacks the side chain at the position C_{13} of the taxane ring, has been identified as a minor metabolite in rat bile.

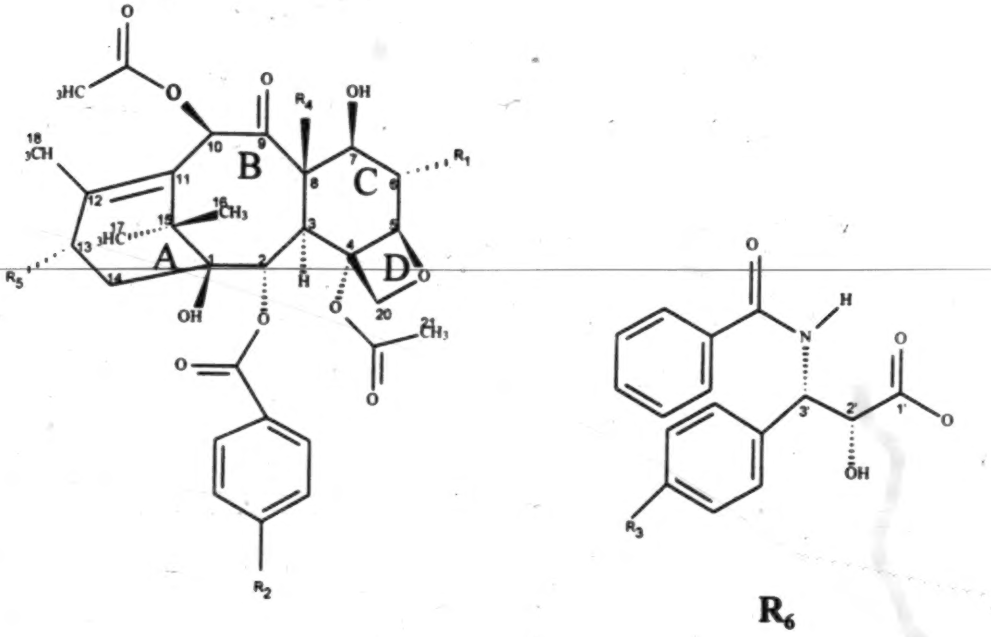

Figure 1-3 **Structures of taxol and biliary metabolites which has been identified in rats (*) and humans (+)**

Table 1-2 **Structures of taxol and biliary metabolites which has been identified in rats (*) and humans (+)**

	R_1	R_2	R_3	R_4	R_5
Taxol	H	H	H	CH_3	R_6
M-1 * +	H	H	H	CH_3	OH
M-2 *	H	OH	H	CH_3	R_6
M-3 *	H	H	H	CH_2OH	R_6
M-4 * +	H	H	OH	CH_3	R_6
M-5 +	OH	H	OH	CH_3	R_6
M-6 +	OH	H	H	CH_3	R_6

With the exception of baccatin III all biliary metabolites identified to date have been hydroxylated derivatives. These metabolites are hydroxylated on side chains at positions

C_2 and C_{13} of the taxane ring as well as on the taxane ring itself. Four of the nine metabolites in rat bile have been spectroscopically identified as monohydroxylated derivatives and one as a dihydroxylated derivative (Table 1-2). Of the five metabolites detected in human bile, two have been identified as monohydroxylated derivatives and one as a dihydroxylated derivative(Figure 1-3). The major metabolite of taxol in human bile, a monohydroxylated derivative with a single hydroxyl group on position C_6 of the taxane ring has not been identified in rat bile. However, the major metabolite in rat bile, a monohydroxylated derivative with a single hydroxyl group on position C_{13} of the taxane nucleus, has only been a minor metabolite in human bile. These metabolites are substantially less active against L1210 and P388 than taxol.

In vitro metabolism studies of taxol using human microsomes have also demonstrated that cytochromes P450 of the CYP2C family are responsible for the formation of the major human hydroxylated metabolite and cytochromes P450 of the CYP3A family are specially responsible for the formation of the metabolite hydroxylated at position C_{13}.

1.2.6 Pharmacokinetics

The pharmacokinetic behavior of taxol has been studied in several phase I clinical trials. Most of these studies have used the longer 6 hr and 24 hr infusion schedules as a result of the high incidence of a major acute hypersensitivity reactions associated with shorter infusions in early clinical trials. Substantial interpatient variability has generally been noted, but there has been no evidence of non-linear or dose-dependent behavior over the relatively broad dose ranges during early studies evaluating prolonged administration schedules.

The mean of peak plasma concentrations of taxol (C_{max}) have ranged from 2.54 to 12.5 μM/L with 3 hr infusions (135-300 mg/m^2); 3.1 to 4.1 μM/L with 6 hr infusions (210-250 mg/m^2); 0.72-0.94 μM/L with 24 hr infusions (210-250 mg/m^2); and 0.053-0.077 μmol/L with 96 hr infusions (120-160 mg/m^2).

In studies of 6 hr and 24 hr administration schedules, both peak plasma taxol concentrations and areas under concentration versus time curves (AUC) have correlated well with taxol dose (Longnecker *et al.*, 1982; Wiernik *et al.*, 1987a,b; Tamura *et al.*, 1993).

The disappearance of taxol from plasma has also been characterized by a biexponential elimination model in most of the early studies. Mean α and β half-lives have ranged from 0.27 to 0.49 hr (cumulative mean 0.34 hr) and 1.3 to 8.6 hr (cumulative mean 4.9 hr), respectively. Mean residence time has ranged from 5.6 to 19.9 hrs (cumulative mean 11.5 hr).

By using more sensitive HPLC assays plasma dispassion data have been well described by a triphasic model with terminal elimination half-lives ranging from 4.6 to 17 hrs (Beijnen *et al.*, 1993; keung *et al.*, 1993; Seibel *et al.*, 1993). Beijnen reported on preliminary pharmacokinetic results using 3 hr infusions and highly sensitive HPLC assay which have shown that the dispassion of taxol in plasma was best described by a three compartment model. The half-lives were 10 minutes ($t_{1/2}\alpha$), 2 hrs ($t_{1/2}\beta$), and 15 hrs ($t_{1/2}\gamma$).

Early results of 3 hr schedule indicate that pharmacokinetics may be non-linear, particularly on shorter schedules, with C_{max} and AUC values increasing disproportionately as the dose increases (Beijnen *et al.*, 1993; Kearns *et al.*, 1993; Keung *et al.*, 1993; Schiller *et al.*, 1993; Seibel *et al.*, 1993). This non-linear profile is important to clinicians. For example, the dose escalations in patients treated on shorter administration schedules will result in disproportionate increases in both AUC and C_{max} along with a disproportionate increase in toxicity. Similarly, dose deescalations may result in disproportionate decreases in AUC and/or C_{max}, potentially decreasing anti-tumor activity.

Mean central (Vd_c) and steady state (Vd_{ss}) volumes of distribution have generally been large, with mean Vd_c ranging from 8.6 to 19.2 l/m^2 (cumulative mean 13.8 l/m^2) and mean Vd_{ss} from 48.2 to 182 l/m^2 (cumulative mean 87 l/m^2, Ohnuma *et al.*, 1985; Longnecker *et al.*, 1986; Grem *et al.*, 1987; Wiernik *et al.*, 1987a,b; Rowinsky *et al.*, 1989; Brown *et al.*, 1991; Forastiere *et al.*, 1992b; Wilson *et al.*, 1992). The Vd_{ss} values are much larger than the volume of total body water, indicating that taxol is extensively bound to proteins and/or other tissue elements, (possibly tubulin and other tissue proteins). Plasma protein binding, as determined by both equilibrium dialysis and ultrafiltration methods, has been reported to range from 95% to greater than 97% over a wide range of drug concentrations (Longnecker *et al.*, 1986; Wiernik *et al.*, 1987a,b). However, despite extensive protein binding to plasma proteins, taxol is readily eliminated from the plasma compartment, suggesting reversible binding of lower affinity.

In early phase I and pharmacological studies of taxol, systemic clearance values have been calculated to range from 100 to 993 ml/min/m^2 (cumulative mean 496 ml/min/m^2). However, the principal mechanisms of systemic clearance have never determined in these early investigations. Total urinary excretion of unmetabolized taxol ranged from 1.4% to 8.2% (cumulative mean 5.5%). These data have suggested that contribution of renal clearance to systemic clearance is minimal, and metabolism, biliary excretion and/or extensive tissue binding probably account for the bulk of the disappearance of the administered dose of taxol.

Eiseeman *et al.*, 1993; Lesser *et al.*, 1993; Klecker *et al.*, 1993 have studied the tissue/plasma distribution ratios of taxol. They showed that tissue/plasma ratios of taxol were high in almost all tissues sampled, including muscle, spleen, liver, lung, and heart. From their results it can be speculated that the ratios were particularly high in tissues that are possibly involved in organ barrier filtration, including the liver and kidney. Blood/brain and blood/testis partition coefficients have been reported to be 0.010 and 0.14, respectively, whereas liver partition coefficients were much greater, 5.24 and 4.07 in males and female mice, respectively (Eiseeman *et al.*, 1993; Lesser *et al.*, 1993; Klecker *et al.*, 1993).

Fujita H. *et al.*, 1994 have studied the bioavailability of taxol after oral administration to ICR mice. Their results showed that AUC(oral)/AUC(iv) ratio in the bile, liver, tumor and plasma was 18.91, 6.71, 0.83 and 0.45%, respectively. On the other hand, Eiseman *et al.* in 1994 defined the pharmacokinetics of paclitaxel after iv, ip, po, and sc administration of 22.5 mg/kg to mice. They concluded that the bioavailability of paclitaxel is approximately 10%, 0, and 0 after ip, po, and sc administration, respectively.

Sparreboom *et al.*, (1996), have shown the contribution of cremophor to the nonlinear pharmacokinetics of paclitaxel in mice. Their results revealed that for a fixed dose of taxol, increasing the amount of cremophor in the formulation, decreases the clearance of taxol. They also illustrated that despite the fact that plasma levels of taxol (for a particular dose of taxol) after administration in cremophor formulation were much higher than tween 80 and dimethylacetamide formulations. However, the level of taxol in tissues was essentially similar with all tested drug preparations.

1.2.7 Pharmaceutics

1.2.7.1 Formulation

Taxol is a relatively non polar compound with a poor solubility in water. Paclitaxel® (the only available formulation of taxol) is formulated as a clear, colorless to slightly yellow, viscous and concentrated solution containing 6 mg/ml of taxol in 50% (v/v) of cremophor EL (polyoxyethylated castor oil) and 50% (v/v) dehydrated alcohol, USP. Taxol is packaged in a single-dose of 5 ml per vial containing 30 mg of taxol. Vials must be diluted prior to use. After dilution with a suitable parenteral fluid (NS, D5W, D5W in NS, and D5W in Ringer's) to 0.3 to 1.2 mg/ml, 135 mg/m^2 taxol is infused intravenously over 24 hrs every 3 week for ovarian cancer or 175 mg/m^2 for breast cancer. Cremophor EL has been observed to cause serious life threating anaphylactoid reactions in animals and humans. Many efforts have been made through chemical modification, to increase taxol's aqueous solubility while retaining cytostatic or antitumor activity, but as of yet these analogous have not clinically replaced the taxol:cremophor formulation. Taxol:cremophor formulation is usually administrated as 3, 6 and 24 h iv infusion but there are some reports that it was administrated as intrapritoneal injection.

1.2.7.2 Stability

The spontaneous conversion of taxol to 7-epitaxol has been shown to occur in normal saline solution at 37° C after 48 hrs. Similar results have been reported in tissue culture medium of J774.2 murine macrophage cells, with approximately 50% of parent drug converted to 7-epitaxol after 72 hrs of drug treatment (Ringel and Horwitz, 1987; Brown *et al.*, 1991). In reverse phase HPLC systems 7-epitaxol elutes after the parent compound. The epimerization of taxol is reversible and 7-epitaxol is also partially converted to taxol under the same conditions. At high temperature and in alkaline solutions taxol undergo hydrolysis to several degraded compounds. The major degraded compounds are bacatin III, 10-deacetyl bactin III followed by 10-deacetyl taxol and 7-epitaxol (Figure 1-4, Ringel and Horwitz, 1987).

It has been observed that over time a particulate matter formed in the diluted solution of cremophor formulation of taxol. This suggests the need for in-line filtration during administration. Nevertheless, the stability study of taxol, which were reported by the NCI in 1983, Waugh *et al.*, and Bristol-Myers Squibb Company, indicated that when taxol was diluted in either NS, D5W, D5W in NS, and D5W in Ringer's solutions to a concentration of 0.03-1.2 mg/ml and passed through a 0.22 µm filter, it had not lost the potency during 27 hr storage of the solution at ambient temperatures (25° C) and normal room lighting conditions.

Cremophor is known to cause the leaching of phthalate plasticizers such as di(2-ethylhexyl)phthalate (DEHP) from polyvinylchloride (PVC) bags and intravenous administration-set tubings. DEHP can cause hepatic toxicity (peroxisome proliferation) in animals, and it is carcinogenic in rodents given high dosages over prolonged periods. The degree of DEHP leaching is directly related to the initial amount of plasticizer in the bags and tubings, the concentration of cremophor EL, and the solution contact time with the plasticized surface. It is therefore, inversely related to the solution flow rate. Waugh *et al.* 1991 has shown that DEHP leaching is not due to the presence of taxol in the infusion solution as the same amount of DEHP is found in solution when the cremophor EL/alcohol vehicle was tested alone. A similar observation has been made about the formulation of intravenous cyclosporine injection, which also contains cremophor EL. They also showed that agitating the bags for 24 hrs had no significant effect on the extent of leaching.

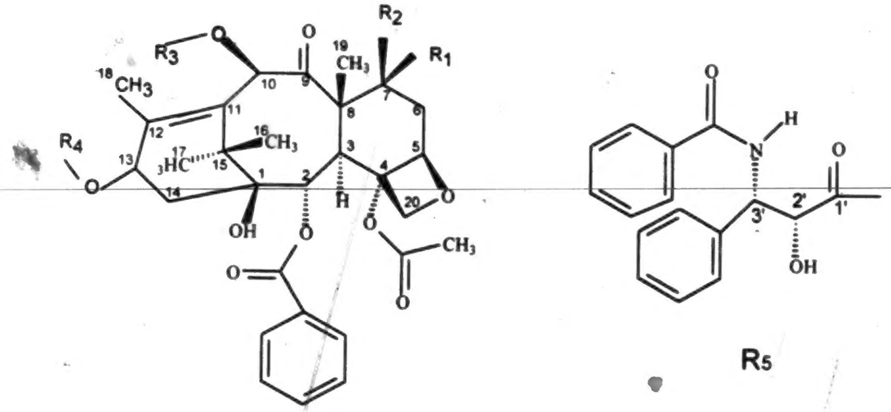
R2
R1
R3
O
19
CH3
18CH3
10
9
7
8
6
11
12
16CH3
R4
13
17
3HC
15
3
5
4
1
2
14
H
20
O
OH
O
CH3
O
O
H
N
O
2'
3'
1'
OH
R5

Figure 1-4 Structure of taxol and its degradation products in alkaline solutions

Table 1-3 Taxol and its degradation products in alkaline solutions

Compound	R_1	R_2	R_3	R_4
Taxol	H	OH	$COCH_3$	R_5
10-deacetyl taxol	H	OH	H	R_5
7-epitaxol	OH	H	$COCH_3$	R_5
10-deacetyl epitaxol	OH	H	H	R_5
Bacatin III	H	OH	$COCH_3$	H
10-deacetyl Bacatin III	H	OH	H	H
Bacatin V	OH	H	$COCH_3$	H
10-deacetyl Bacatin V	OH	H	H	H

1.2.7.3 Toxicity of Cremophor EL

Acute allergic reactions have been reported in 1-5 percent of patients treated with cremophor formulation of taxol, tenoposide, and cyclosporine. These reactions which occurred immediately after the initiation of the infusion, consisted of shortness of breath, flushing, tachypnea, chest pain, pruntus, or urticaria.

Other effects of cremophor EL injection have been studied in dogs. The administration of a daily intravenous infusion of the surfactant at a dose of 0.5 ml/kg produced symptoms usually indicative of an allergic reaction, including flushing of the skin, penorbital edema, and shaking of the head during or shortly after infusion. Increases in platelet count and serum lipid values, including total cholesterol, triglycerides, and total lipids, occurred as the daily administration of cremophor EL continued for up to 30 days. In contrast, there was a decrease in the percentage of alpha-lipoproteins in the blood. Histopathologic examination revealed increased tissue lipids in the spleen and the lymph nodes (Howric DL *et al.* 1985).

1.2.7.4 Cremophor EL and Multidrug Resistance

Mammalian cells that exhibit the MDR phenotype produce high concentrations of proline-glycoprotein (P-glycoprotein), a membrane protein that acts as a broad-spectrum pump to remove a number of natural-product antineoplastic drugs, including the anthracyclines (doxorubicin and daunorubicin) and the vinca alkaloids (i.e., vinblastine, vincristine) from the cell. More recently, taxol also has been shown to induce the MDR phenotype. A number of agents have been found to reverse MDR, but their use has been restricted because of dose-limiting toxicities. Cremophor EL, which lacks direct antitumor activity, reverses the MDR phenotype in cells in culture at concentrations likely to be readily available clinically. In an MDR neuroblastoma cell line with known resistance to homoharringtonine and doxorubicin, cremophor EL reversed a 214-fold resistance to taxol that was observed when this agent was dissolved in a vehicle not containing cremophor EL. When cells were treated with cremophor EL at the same concentration as that used to dissolve taxol, the surfactant had no cytotoxic effect, suggesting that it acts by reversing resistance rather than by directly killing cells.

In 1992, the capability of a variety of surface-active agents was compared with that of cremophor EL. Several surface-active agents, including some that are used as pharmaceutical adjuvants, were compared with cremophor EL. Although many of the agents, including Triton X-100 and Tween-80, showed a similar level of activity in reversing MDR, as indicated by an increased equilibrium concentration of daunorubicin in R100 cells, cremophor EL had the lowest inherent cytotoxicity. The only detergents capable of reversing MDR were those containing a polyethylene oxide hydrophilic moiety. The equilibrium concentration of daunorubicin in R100 cells in the presence of cremophor EL is concentration-dependent, reaching a maximum that is equivalent to the level in sensitive CEM cells at a 1:1000 dilution (Woodcock DM *et al.* 1992). Cells recover rapidly from cremophor EL's reversal of daunorubicin exclusion. One hr after the detergent was removed, the R100 cells had recovered the capability to extrude drug. The uptake and efflux of daunorubicin by CEM, and R100 cells are affected by the presence of cremophor EL. Uptake in both cell types was reduced initially, but total uptake continued to increase in the MDR cell line. Thus, the primary effect of cremophor EL appears to be a decrease in the capability of resistant cells to actively efflux the drug from R100 cells, which implies that the surfactant is blocking the activity of the P-glycoprotein (Koller and Dorr, 1994).

Although cremophor EL can reverse MDR *in vitro* and *in vivo*, relatively high concentrations of cremophor EL are required for resistance modulation (0.375 mM). These concentrations are much higher than those that would be achieved by administering taxol at a dose of 135 mg/m^2. Blocked drug efflux and enhanced membrane fluidity by cremophor EL are rapidly reversible and do not involve cell lysis. Enhanced membrane fluidity may disable the capability of the P-glycoprotein to expel the drug. Cremophor EL does not appear to be a major vesicant, although the complete formulation may have vesicant activity.

1.3 Cyclodextrins

Cyclodextrins are oligomers of glucose which are produced by enzymatic (cyclodextrin transglycosylase, CTG) degradation of starch. Starch consists of two main polymeric structures, branched amylopectin and linear amylose. The building blocks of these poly-sugars are D-glucopyranoside units linked by (X-1,4 and X-1,6 glycosidic bonds). The partial degradation of amylopectin and amylose results in heterogeneous, water absorbing, and water-soluble compounds called dextrins. Further simultaneous enzymatic degradation and the attachment of two ends of the dextrin molecules produces a cyclic oligosaccharide, cyclodextrin (Szejtii, 1982 and 1994). Cyclodextrins are classified by the number of α-1,4-linked glucose units which occur in their molecular structure. αCD has six such units, βCD has seven, and γCD has eight (Figure 1-5). In these compounds, the C-1 chain conformations of the glucose monomers arrange the molecule in a cone-like structure in which the hydroxy groups are oriented towards the exterior. The narrower end of the cone contains the primary hydroxy functionality while the secondary hydroxyl groups are situated towards the wider end (Figure 1-6). This arrangement makes the cyclodextrin exterior predominantly hydrophilic. The secondary hydroxyl groups are able to interact via hydrogen bonding to stabilize the crystalline lattice. This reduce to a large extent the solubility of cyclodextrins, especially βCD, in water. The interior of the cyclodextrin cone is predominantly hydrophobic because of the assembly of the skeletal hydrocarbon chains. Hence, the interior of the cone structure of cyclodextrin represents a non-polar environment suitable for solubilizing poorly water-soluble compounds (Szejtii, 1982 and 1994).

1.3.1 Derivatives

Several cyclodextrin derivatives have been prepared in order to improve: 1) the solubility of the parent cyclodextrins; 2) the solubilizing potential of cyclodextrins; 3) the stability of inclusion complexes; 4) reducing the toxicity of cyclodextrins; 5) attachment of catalytic groups (enzyme modelling); 6) production of less soluble or insoluble derivatives of parent molecules for sustained release formulation or chromatographic purposes. Hydrogen or hydroxyls atoms of cyclodextrins can be substituted with a variety of groups such as alkyl, hydroxyalkyl, and carboxyalkyl moieties using chemical or enzymatic reactions, (Irie *et al.* 1989, Lindberg *et al.* 1991). Although many different derivatives of cyclodextrins have been prepared (Table 1-4), the pharmaceutical applications of these derivatives have been limited due to lack of safety data on these derivatives (Szejtli, 1994). Only a few, methylated and hydroxypropylated cyclodextrins have been widely used for pharmaceutical purposes.

Dimethyl-β-cyclodextrin (DMβCD) and hydroxypropyl-β-cyclodextrin (HPβCD) have been extensively studied as solubilizing agents for poorly water-soluble drugs (Szejtli, 1994). Considerable attention has been paid to the utilization of the HPβCD derivative in various pharmaceutical formulations, particularly as a solubilizing agent in parenteral formulations (Yoshida *et al.* 1989).

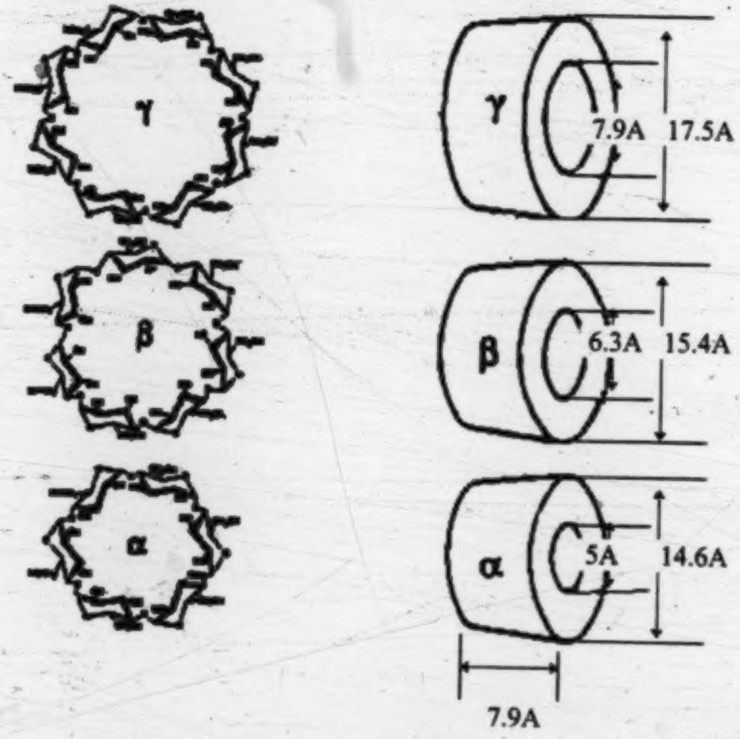

Figure 1-5 **Molecular structure and dimensions of cyclodextrins**

1.3.2 Physicochemical Properties

Table 1-4 depicts some of the physicochemical properties of various cyclodextrins (Szejtii, 1982 and 1994). The aqueous solubilities of parent cyclodextrins are temperature dependent. Cyclodextrins (αCD, βCD, and γCD) are not sensitive to alkali hydrolysis and have no well defined melting point. They start to decompose above 200° C and are completely decomposed at 250° C. Cyclodextrins partially hydrolyse to glucose units and linear oligosaccharides in strong acid solutions (e.g., 1-5 M HCl).

Table 1-4 **Physicochemical properties of some cyclodextrins**

Physicochemical properties	α-cyclodextrin	β-cyclodextrin	γ-cyclodextrin	HPβCD
Number of glucose units	6	7	8	7
Average Molecular Weight	972	1135	1279	1384
Solubility g/100 ml	14.5	1.8	23.2	>50
Primary hydroxyl group C (6)	H	H	H	$-CH_2-CHOH-CH_3$
Secondary hydroxyl group [C (2), C (3)]	H	H	H	$-CH_2-CHOH-CH_3$

1.3.3 Toxicity

To the best of our knowledge there is no report on any apparent damage in the kidneys, GI tract, central nervous system, and/or blood cells after oral administration of cyclodextrins. This is probably due to lack of absorption of intact cyclodextrins and their derivatives from the GI tract. In contrast to oral administration, however, renal damage is the main toxicity following iv administration of β-Cyclodextrin due to a particularly high affinity for cholesterol (Szejtli, 1982). Parenterally administered cyclodextrin forms an insoluble cholesterol-β-cyclodextrin complex in the kidneys causing nephrotoxicity. Methylated β-cyclodextrin-cholesterol complexes are more stable than the normal β-cyclodextrin-cholesterol complexes. Methyl-β-cyclodextrin is capable of extracting cholesterol from the erythrocyte membrane often resulting in extensive hemolysis. Nephrotoxicity is significantly reduced or abolished with modified cyclodextrins with higher water solubility such as HPβCD (Szejtli, 1982 and 1994 and Yoshida *et al.* 1989).

1.3.4 Inclusion Complexes with Drugs

Inclusion complex or molecular encapsulation is a product of encapsulation of a molecule (guest) in a cavity of a second molecule (host). The unique cone shape structure of cyclodextrins enables them to form a host-guest complex (Figure 1-6). The exterior of the cyclodextrin cone is hydrophilic while due to the relatively high electron density its cavity is hydrophobic. In an aqueous environment the hydrophobic cavity of the cyclodextrin molecule is occupied by water molecules. The interaction of water with the cavity of the ß-cyclodextrin is thermodynamically undesirable due to polar-apolar interactions. Water molecules can readily be replaced by more hydrophobic drug molecules resulting in the formation of cyclodextrin-drug inclusion complexes. This characteristics makes cyclodextrins water-soluble compounds which can form soluble inclusion complexes with poorly water-soluble molecules. In the cyclodextrin complexation, the major consideration is that some large and complex molecules can not fit wholly into the cyclodextrin cavity. But depending on the type of the utilized cyclodextrin, at least some part of a molecule can fit into the cyclodextrin cavity. One of the expected improvements provided by the complex is an increase in aqueous solubility. The formation of an inclusion complex is confirmed by X-ray crystallography, spectroscopic methods (IR, MS, and NMR spectroscopy), chromatography (thin-layer), solubility studies, and thermal analysis with a differential scanning calorimeter (DSC, Szejtli, 1982 and 1994).

Characteristics of the inclusion complexes formed between cyclodextrins and drugs can be determined by examining the effect of solubilizer concentration on the drug solubility. According to the definition defined by Higuchi and Connors, the two main types of solubility profiles, A and B, can be seen for cyclodextrin complexation. A-type curves indicate the formation of soluble inclusion complexes, while B-type relationships indicate the formation of complexes with limited solubility.

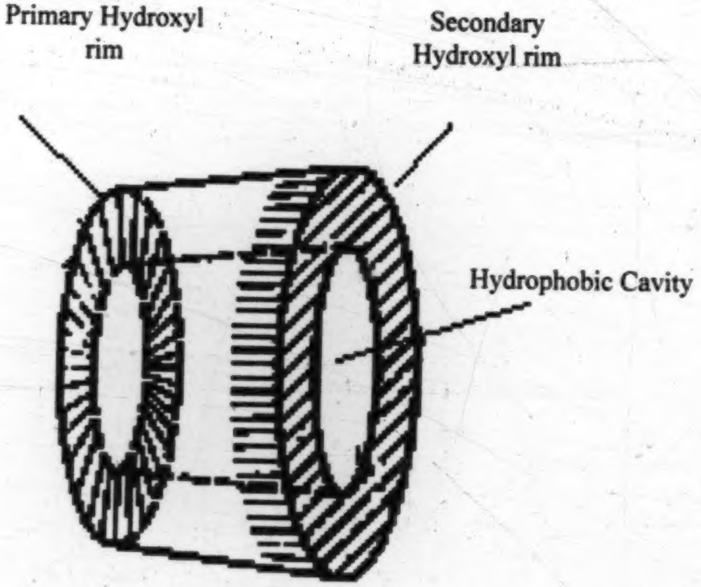

Figure 1-6 Basic feature of cone shape structure of cyclodextrins

1.3.5 Preparation of Cyclodextrins Inclusion Complexes

The preparation of inclusion complex of drug molecules with cyclodextrins is a simple process. The most common methods of preparation are: phase solubility, sonication, solvent evaporation, dialysis, and kneading methods.

1.3.5.1 Phase Solubility Method

In this method an aqueous solution of cyclodextrin is stirred or shaken with solid particles or an aqueous solution of drug. Following establishment of equilibrium (several hrs to several days) and separation of uncomplexed drug, water can be removed by freeze-drying, spray drying or by any other suitable methods.

1.3.5.2 Sonication Method

In this method a mixture of aqueous solution of cyclodextrin with solid particles of drug are sonicated for several min to several hrs. Following establishment of equilibrium and separation of the uncomplexed drug, water can be removed by freeze-drying, spray drying or by any other suitable method. Due to the high energy of sonication, the particle size decreases, the connection of cyclodextrin and drug particles improves, therefore, the possibility of favourable interaction between drug and cyclodextrin is increases and the inclusion complex with drug is achieved faster.

1.3.5.3 Solvent Evaporation Method

In this method an ether solution of drug is added gradually to an aqueous solution of cyclodextrin while the system is connected to vacuum and shaken well. Following a complete removal of ether and establishment of equilibrium and separation of the uncomplexed drug, water can be removed by freeze-drying, spray drying or by any other suitable methods. Because of the production of small particle size emulsions and possibly suspensions, the connection of cyclodextrin and drug particles improves therefore, the possibility of favourable interaction between drug and cyclodextrin increases and the inclusion complex with drug is obtained faster.

1.3.5.4 Dialysis Method

This method is used for bulky structural compounds (MW > 500). In this method cyclodextrin and drug are dissolved in ethanol, DMSO, DMF, or any other water-miscible low molecular weight solvent, and then transferred to a dialysis bag (MW > 500). After dialyzing (molecular weight cutoff < 500 g/mol) against distilled water at constant temperature and establishment of equilibrium the product is collected, uncomplexed drug separated, the water is removed by freeze-drying, spray drying or by any other suitable methods.

1.3.5.5 Kneading Method

Another method for the preparation of an inclusion complex as a solid product is kneading. This method involves kneading cyclodextrin and the drug with a small amount of water. Since the cyclodextrin-water interaction is thermodynamically unfavourable, the inclusion complex with drug is easily obtained.

1.3.6 Change in Physicochemical Properties of Drugs

Cyclodextrins, by virtue of their ability to form inclusion complexes with many drugs, can substantially change the physicochemical properties of pharmaceuticals.

1.3.6.1 Solubility and Dissolution

In an aqueous environment the hydrophilic exterior part of the cyclodextrin molecule comes in direct contact with water molecules. This interaction results in an increase in water solubility of the hydrophobic guest molecule. Following inclusion complexation with anticancer drugs, a similar improvement in the solubility and dissolution characteristics of these drugs have been reported.

The interaction between 23 anticancer drugs and carboxymethyl-gamma-cyclodextrin (CM-γCD) were studied by Cserhati *et al.* (1994 and 1995). CM-γCD formed inclusion complexes with 11 of those compounds. The complexes were always more hydrophilic than the uncomplexed drug. The inclusion-forming capacity of a drug differed considerably depending on its chemical structure. Torres-Labandeira *et al.* (1991), evaluated the effect of 15 cyclodextrin derivatives on the solubility of pancratistatin. Solubility of pancratistatin in water is 50 μg/ml and cyclodextrins increased the solubility of this compound in water to 0.1-1.2 mg/ml. Complexes of pancratistatin with hydroxypropyl-beta-cyclodextrin were more stable than those with hydroxypropyl-gamma-cyclodextrin. Psoralen was found to form an inclusion complex with beta-cyclodextrin (βCD), heptakis(2,6-di-O-methyl)-beta-cyclodextrin (DMβCD) and heptakis(2,3,6-tri-O-methyl)-beta-cyclodextrin (TMβCD). Phase solubility studies revealed the formation of a 1:1 molar complex. The solubility and dissolution rate of the complexed forms were improved, particularly for the DMβCD complex (Vincieri *et al.* 1995). Arcari *et al.* (1992), showed a dramatic increase in the dissolution rate of silymarin, by complexation with βCD. Following inclusion complexation with cyclodextrins, a similar improvement in the solubility and dissolution characteristics of some of the nonsteroidal anti-inflammatory drugs (NSAIDs) have been reported. The net effect of complexation of the NSAIDs with hydrophilic cyclodextrin derivatives is to enhance water solubility and dissolution rate of these drugs (Masuda *et al.* 1984, Tarimci and Celebi, 1988, Uekama *et al.* 1981 and 1985, Zecchi *et al.* 1988, and Loftsson *et al.* 1993). However, the hydrophobic derivatives of cyclodextrin have been used to prepare sustained release oral formulations (Vakilynejad *et al.* 1996). For example, following complexation with dimethylcyclodextrin, the release rate of diltiazem has been significantly reduced (Horiuchi, 1990).

1.3.6.2 Stability

Molecular encapsulation of drugs by cyclodextrins can substantially increase the stability of compounds. The ability of cyclodextrin to encapsulate drug molecules makes them valuable agents for improvement of the physical and chemical stability of unstable drug molecules. Cyclodextrin can decrease or increase various kinds of reactions in drugs solutions. This effect depends on the structure and concentration of cyclodextrin, as well as solubility and stability of the drug-cyclodextrin complex. It has been suggested that the inclusion of drug molecules in cyclodextrins may inhibit the attack of degrading agents such as H^+, OH^-, O, light, or water molecules and thus decreases the rate of hydrolysis, oxidation, proteolysis, epimerization, racemization or other decomposition reactions.

The effects of cyclodextrins on the chemical stability of several mitomycin antibiotics in an alkaline medium have been investigated by Bekers *et al.* (1991). They showed that complexation is most favorable with gamma-cyclodextrin. All mitomycin-gamma-cyclodextrin complexes degrade at lower rates than those of the free drugs. Banga *et al.*, (1993), have reported the efficacy of cyclodextrins in preventing aggregation of insulin where the best effect was observed for HPβCD followed by βCD, and αCD. No protection was observed with γCD. Loftsson, (1992) investigated the effects of six cyclodextrin derivatives on the stability of tauromustine in aqueous buffer solutions. All the CDs tested had some stabilizing effect on the drug at pH 1.98 and 4.06 but they had little or no effect at pH 6.3-8. Also improvement of stability of prostaglandin E1(Yamamoto *et al.* 1992), psoralen (Vincieri *et al.* 1995), nitrazepamm (Saleh SI., *et al.* 1993), 2', 3'-dideoxyinosine (Bekers *et al.* 1993), thalidomide (Krenn *et al.* 1992), Benzylpenicillin (Pop *et al.* 1991), Naproxen (Frijlink *et al.* 1991) flurbiprofen (Frijlink *et al.* 1991), S-nitroso N-acetyl penicillamine (Bauer *et al.* 1991), Leucine enkephalin (Irwin *et al.* 1994), biphenylylacetic acid (Arima *et al.* 1992), decrease in chemical racemization of both (-)-Shyoscyamine and (-)-S-scopolamine (Blaschke *et al.* 1993) by cyclodextrins complexation have been reported.

1.3.6.3 Bioavailability

The effects of cyclodextrins on the cellular, nasal, transdermal, corneal, and oral, absorption of drugs have been investigated. It has been reported that complexation with cyclodextrins can enhance the oral bioavailability (Ricevuti *et al.*, 1991, Merkus *et al.* 1991, Betlach *et al.*, 1993, Arcari *et al.* 1992, Shao *et al.*, 1994, Uekama *et al.* 1992 and 1995, Woodcock *et al.* 1993, Deroubaix *et al.*, 1995), nasal bioavailability (Watanabe *et al.* 1993, Schipper *et al.* 1993 and 1995), ocular bioavailability (Usayapant *et al.* 1991, Freedman *et al.* 1993, Jarvinen *et al.* 1994, Suhonen 1995), rectal bioavailability (Arima *et al.*, 1992 Uekama *et al.* 1995) and sublingual (Stuenkel *et al.* 1991) bioavailability of drugs.

Cyclodextrins by their ability to make complex with compounds can change the bioavailability of the drugs by different mechanisms, depending on the route of administration and physicochemical properties of the drug, cyclodextrin, and cyclodextrin-drug complex. Improvement in bioavailability of silybinin (Arcari *et al.* 1992), piroxicam (Woodcock *et al.*, 1993, Deroubaix *et al.* 1995), and nifedipine (Uekama *et al.*, 1992), has been reported to be due to better solubility and faster dissolution rate of the drug-cyclodextrin inclusion complex.

Cyclodextrins may act as absorption enhancer by changing the structure of the cell membranes or influencing the partitioning behavior of drug in the cell membrane (Vollmer *et al.* 1994). Schipper *et al.* (1993) reported the reversible increase in nasal permeability of Org276 (a metabolically stabilized ACTH analogue) by DMβCD in rat and rabbit. Transdermal penetration enhancement of the cytochrome P450 inhibitor liarozole by HPβCD and DMβCD were reported by Vollmer *et al.* in 1994. They concluded that their results reveal that DMβCD acts as a transdermal absorption enhancer by changing the stratum corneum barrier whereas HPβCD influences the partitioning behavior of the drug in the skin. Tous *et al.*, (1992), studied the availability of phenindione-β-cyclodextrin inclusion complex. Their results revealed that the permeability coefficients of phenindione increased to 157% in the presence of β-cyclodextrin in the formulation either by complexation or physical mixing. They showed that the presence of human albumin generally decreases the permeability coefficient of the drug. Muller *et al.*, in 1991 have shown that competitive displacement of the inclusion molecule from its HPβCD complex by sodium deoxycholate and cholesterol participates in the release mechanism of the inclusion molecule under *in vivo* conditions.

The effect of cyclodextrins on the cell membranes is dependent on the cyclodextrin structure and concentration. Merkus *et al.*, in 1991 have shown effect of cyclodextrins on intranasally administered insulin in rats. They concluded that DMβCD is a potent enhancer of nasal insulin absorption in rats. The other CDs, HPβCD (5%), βCD (1.8%), and γCD (5%), did not have significant effects on nasal insulin absorption.

Shao *et al.*, 1992 have investigated the safety and effectiveness of cyclodextrins as nasal absorption promoters of peptide-like macromolecules. They found that the relative effectiveness of the cyclodextrins in enhancing insulin nasal absorption to be in the descending order of DMβCD > αCD > βCD > HPβCD > γCD. A direct relationship linking absorption promotion to nasal membrane protein release is evident, which in turn correlates well with nasal membrane phospholipid release. The magnitude of the membrane damaging effects determined by the membrane protein or phospholipid release provided an accurate, simple, and useful marker for predicting safety of the absorption enhancers. The magnitude of damage and specificity of cyclodextrin derivatives in solubilizing nasal membrane components, has been determined by measuring the enzymatic activities of membrane-bound 5'-nucleotidase (5'-ND) and intracellular lactate dehydrogenase (LDH) in the perfusates. HPβCD at a 5% concentration has been found to result in only minimal removal of epithelial membrane proteins as evidenced by a slight increase in 5'-ND and total absence of LDH activity. On the other hand, 5% DMβCD has caused extensive removal of the membrane-bound 5'-ND. Moreover, intracellular LDH activity in the perfusate increased almost linearly with time.

Histopathological examination of the rat intestine has been performed by Shao *et al.*, (1994), to detect tissue damage following enteral administration of the β-cyclodextrin derivatives. Light microscopic inspection indicated no observable tissue damage. This study indicates the feasibility of using cyclodextrins as mucosal absorption promoters of proteins and peptide drugs

1.3.6.4 Side Effects

The complexation of drugs with cyclodextrin may also decrease the side effects of drugs such as indomethacin, flurbiprofen, biphenylacetic acid, naproxen, and phenylbutazone. These are examples of drugs with GI mucosal irritating effects that may be reduced by cyclodextrin complexation (Szejtli, 1994, Rainsford, 1990, Lee *et al.* 1994). Overall, molecular encapsulation using cyclodextrins may result in better oral absorption, better bioavailability, less interindividual variability, less side effects and stronger biological activities (Hoshino *et al.* 1989, Fregnan *et al.* 1990), for some drugs in animals and humans.

1.3.7 Other Pharmaceutical Application of Cyclodextrins

1.3.7.1 As Excipients

The potential application of cyclodextrins in the formulation of parenteral, oral, rectal, bucal, sublingual, epidural, intrathecal, pulmonary, transdermal, and nasal drug delivery systems is increasing (Szejtli, 1994). In tableting cyclodextrins (βCD) can be used as an ingredient to increase stability or solubility of active ingredient. Also it can be added as a filler and/or binder to improve compression properties of powder blend. Cyclodextrins have been studied as excipients in the tablet formulations of many therapeutically important drugs such as acetylsalicylic acid, ergocalciferol, isosorbide 5-monohydrate, phenytoin, sulfamethoxazole, and furosemide (Szejtli, 1994).

1.3.7.2 Role in the Separation of Enantiomers

Cyclodextrins are optically pure compounds. The drug molecules can fit in the cavity of the cyclodextrins and resolution occurs due to formation of bonds (hydrogen, hydrophilic etc) between the substituted groups on the outside of the cyclodextrin cavity and the drug molecule. Due to additional electrostatic interactions between the chiral drug and the groups at the entrance of the cyclodextrin cavity, modified cyclodextrins may exhibit a higher degree of chiral selectivity (Imai 1988, 1995a, and 1995b). Cyclodextrins may also be used for stereoselective release of drugs. The results of Zecchi *et al.* (1988), and Orienti *et al.* (1989), have shown that when cyclodextrins are used in the formulation of racemic drugs, the possibility of stereoselective release and subsequent absorption of the enantiomers may occur, especially from drug-cyclodextrin complexes (such as drug-methyl-CD complex) with slow dissolution rate.

1.4 Nicotinamide

Nicotinamide, (Figure 1-7, niacinamide, nicotinamide, or 3-pyridinecarboxamide), is a nontoxic B vitamin (vitamin B_3), which is freely soluble in water, ethanol, and glycerol, and poorly soluble in chloroform and ether. In aqueous solution nicotinamide is almost neutral in reaction. As a base nicotinamide forms salts, e.g., with hydrochloric acid. Nicotinamide exhibits a relatively high degree of thermal stability. The optimum stability is at pH values between 4.5 and 6.0. However, in strongly acidic or strongly alkaline solution, hydrolysis occurs. Nicotinamide is able to form intermolecular hydrogen bonding (Roth *et al.* 1994).

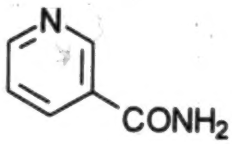

Figure 1-7 Structure of nicotinamide

Nicotinamide has been shown to enhance the aqueous solubility of many drugs through complexation. The exact mechanism by which this compound form complexes is not entirely clear. The solubility enhancement of steroids (Samejima *et al.*, 1960), menadion (Hata *et al.* 1970), Moricizine (Hussain *et al.*, 1992), and certain heteroaromatic drug molecules (Fawzi *et al.* 1980) by complex formation with nicotinamide were explained as a result of a *Pi*-donor *Pi*-acceptor interaction. Hamza and Parrots (1985) studied the increase in the aqueous solubility of acetaminophen in the presence of nicotinamide and other hydrotrops as solubilizing agents. They concluded that the enhancement was mediated through hydrotrophy, although there are some more evidences from UV and TLC analysis that nicotinamide and acetaminophen enter into complex formation.

1.4.1 Pharmaceutical Application

Nicotinamide has been reported to increase solubility, stability, and dissolution of compounds. The enhancement of solubility of moricizine complexation with nicotinamide was examined by Hussain *et al.*, 1993 as a potential solubilization technique. Muller *et al.*, in 1991 showed the effects of nicotinamide as a hydrotropic substances on the phase solubility inclusion complex formation between methyltestosterone and HPβCD. Rasool *et al.*, (1991) studied the solubilities of five poorly water-soluble drugs, diazepam, griseofulvin, progesterone, 17 beta-estradiol, and testosterone, in the presence of nicotinamide. All solubilities of all of mentioned drugs was found to increase in a nonlinear fashion as a function of nicotinamide concentration. Naggar *et al.*, in 1980, have shown that nicotinamide can increase the dissolution of nitrofurantoin in either water or Sorensen's phosphate buffer (pH 5). Dissolution rate studies by El-Banna *et al.* (1977), revealed that the fused nicotinamide/caffeine (50:50 and 70:30) solid dispersions exhibited better (3.7 fold faster) dissolution rate than that of the pure drug. Cannon *et al.* (1995) reported that niacinamide can stabilize hemin, apparently by complexing to hemin and preventing the formation of hematin dimers.

Nicotinamide is given as oral doses of 3, 4, 5, 6 and 10 g to patients who are undergoing treatment with hyperthermia and radiation therapy for a variety of recurrent/metastatic cancers. (Hoskin *et al.* 1995, Cartei *et al.* 1994, Rojas 1993). Also nicotinamide is currently under trial for the prevention of insulin-dependent diabetes mellitus (Petley *et al.* 1995). Different formulations of nicotinamide, such as standard and the long-acting Enduramide, are available.

1.4.2 Preparation of Inclusion Complexes

The preparation of inclusion complex of drug molecules with nicotinamide is a simple process. The most common methods of preparation are: phase solubility, sonication, solvent evaporation, and kneading methods

1.4.2.1 Phase Solubility Method

In this method an aqueous solution of nicotinamide is stirred or shaken with solid particles or an aqueous solution of drug. Following the establishment of equilibrium (several hrs to several days) and separation of uncomplexed drug, water can be removed by freeze-drying, spray drying or by any other suitable method.

1.4.2.2 Sonication Method

In this method a mixture of aqueous solution of nicotinamide with solid particles of drug is sonicated for several min to several hrs. Following the establishment of equilibrium and separation of uncomplexed drug, water can be removed by freeze-drying, spray drying or by any other suitable method. Due to the high energy of sonication, the particle size is decreased and the connection of nicotinamide and drug particle is improved. Therefore, the possibility of favourable interaction between drug and nicotinamide is increased and the inclusion complex with drug is achieved faster.

1.4.2.3 Solvent Evaporation Method

In this method an ether solution of drug is added gradually to an aqueous solution of nicotinamide while the system is connected to vacuum and shacked well. Following the complete removal of ether and establishment of equilibrium and separation of uncomplexed drug, water can be removed by freeze-drying, spray drying or by any other suitable method. Because of the production of small particle size emulation and possibly suspension, the connection of nicotinamide and drug particles are improved therefore, the possibility of favourable interaction between drug and nicotinamide is increased and the inclusion complex with drug is obtained

1.4.2.4 Kneading Method

Kneading is an alternative method for the preparation of an inclusion complex as a solid product. This method involves kneading nicotinamide and the drug with a small amount of water. Since the nicotinamide-water interaction is thermodynamically unfavourable, the inclusion complex with drug is easily obtained.

1.4.3 Clinical Application

Nicotinamide, is currently under clinical trial for the prevention of insulin-dependent diabetes mellitus after success in the NOD mouse (Stratford *et al.* 1994, Elliott *et al.* 1993. Kallmann *et al.* 1992). Also, nicotinamide has been shown to act as a radiosensitizer drug in experimental rodent tumors growing in skin and muscle while producing little or no radiosensitization of normal tissues (Brown *et al.* 1991, Lee *et al.*, 1992, Ono *et al.* 1993, Horsman *et al.* 1993). Its mechanism of action is different from classical electron-affinic compounds and appears to be the result of improved tumor oxygenation, reduction in the mean arterial blood pressure and decreased flow resistance in tumors, which would lower vascular pressure and tumor interstitial fluid pressure (TIFP).

Chaplin *et al.* (1991) has illustrated that a combination of nicotinamide and fluosol DA/carbogen can provide an effective way of reoxygenating in both acutely and chronically hypoxic cells. Some results (Lee *et al.*, 1992) have shown that nicotinamide causes approximately a 15% decrease in mean arterial blood pressure ($P < 0.05$) and a 35% decrease in TIFP (P less than 0.001) at 2 h postinjection without any change in hematocrit. The change in TIFP has been found to be tumor size dependent. Also Hirst *et al.* 1993, have shown that nicotinamide causes an increase (17-92%) in cardiac output distribution (COD) of an experimental drug to tumors mouse carcinoma growing in skin, muscle or the gut wall. They concluded that nicotinamide alters COD to tumors in some sites relative to host tissues in a way that could enhance anti-cancer drug delivery to tumors. If these results could be confirmed in human tumors in the near future, they would have significant implications in cancer treatment using radiation, chemotherapy, and immunotherapy.

1.4.4 Pharmacokinetics

In mice, nicotinamide given ip at doses of 100-500 mg/kg showed biphasic elimination with dose-dependent changes in half-life. Nicotinamide N-oxide is the main plasma metabolite in mice. The N-oxide, which is also a weak radiosensitizer, is subject to reduction to the parent nicotinamide following administration at a dose of 276 mg/kg. The bioavailability of both drugs given via the ip, as compared with the iv route was close to 100%. Tumor concentrations of nicotinamide paralleled those in the plasma after a short lag. Tumor nicotinamide adenine dinucleotide (NAD) concentrations were elevated by factors of 1.5 and 1.8 following doses of 100 and 500 mg/kg nicotinamide, respectively. The dose, route of administration, and formulation of nicotinamide given to humans are quite different from those used successfully in animals. The average peak plasma concentration of 160 μg/ml measured in humans after taking 6 g of nicotinamide was equivalent to that seen in mice after injecting 171 mg/kg.

1.5 Liposomes

Liposomes are self-assembling colloidal particles in which a lipid bilayer encapsulates a fraction of the surrounding aqueous medium. Liposomes are composed of phospholipid and form unilamellar or multilamellar concentric bilayers separated by aqueous compartments. Thirty one years ago Alec Bangham discovered that phospholipids in water form closed vesicles (Bangham 1965); the physicochemical properties of these liposomes were then characterised, including their ability to serve as a model for cell membranes and as an important drug delivery system (Fildes, 1981). Because of the structural similarity between liposome and cell membrane, liposomes may be used to entrap, sustain, protect, and transport, both polar and nonpolar drug molecules to cells or specific cell lines.

Initially liposomes were introduced as optimal drug carrier systems, but further research proved that they have large uncontrollable properties upon administration *in vivo*. After *in vivo* administration, liposomes are susceptible to the changes in pH, catalytic activity of serum proteins [high density lipoproteins (HDLs)], bile salts, and various proteases and peptidases (Lasic 1995, Richards 1978 and Op Den Kamp 1974). Liposomes administered iv are cleared from systemic circulation by phagocytic cells of the immune system, predominantly in liver and spleen due to non-specific reactivity of conventional liposomes (this property led to some clinically important applications of liposomes in antiparasitic treatment of phagocytes and in vaccine formulations).

In recent years, considerable attention has been paid to the rationally designed liposomes (sophisticated liposomes), resulting in nonreactive (sterically stabilized) liposomes, specifically reactive liposomes (targeting), and highly reactive liposomes (polymorphic liposomes or liposomes which can exhibit high reactivity to nucleic acids and cell membranes). Due to their reduced recognition and uptake by the immune system, these newly sophisticated liposomes have been referred to as "stealth" liposomes, and are proving to be useful in cancer chemotherapy. The polymorphic liposomes provide a promising approach to gene therapy because they greatly improve transfection by erogenous DNA (Lasic and Papahadjopoulos 1995).

In parallel with these developments, more efficient loading and retention of drugs within liposomes has contributed to the ultimate utility of this new generation of liposomes in improving the toxicological profile of drugs and delivery of anticancer agents, antifungal drugs, antibacterials, antivirals, and antiparasitics (Cullis, 1987, Lichtenberger, 1988 Fielding 1991, Lasic 1995).

1.5.1 Preparation of Liposomes

Liposomes can entrap both hydrophilic and hydrophobic compounds. In general, hydrophobic compounds tend to remain in the phospholipid bilayer while hydrophilic molecules reside in the aqueous compartment of the liposomal vesicles. Liposome characteristics such as gel-to-liquid crystalline transitions, permeability, partition coefficients, electrical properties, and elastic properties are important parameters in quality and quantity of drug delivery by liposomal systems. These properties are altered by using mixtures of phospholipids (neutral and charged), including or excluding cholesterol, or charged adducts in the formulation of liposomes (Gruner, 1987). Liposomes may be prepared from phospholipids using a number of techniques such as hydration, reverse-phase evaporation, detergent removal, and solvent injection methods. In general, the method used in the preparation of liposomes determines their size and type, and loading capacity.

1.5.2 Stability of Liposomes

At high temperatures and at extremely high pH values, phospholipids undergo hydrolysis of the fatty acid attached to the glycerol moiety, and produce lysophospholipid and free fatty acid. The formation of such hydrolysis side products significantly decrease the stability of the liposome (Grit *et al.* 1989).

Liposomes may be degraded because of a change in pH or interaction with bile salts, plasma proteins (such as opsonins and lipoproteins) and cell surface receptors. In order to enhance biological stability of liposomes, lipid bilayers containing glycolipids or lipids conjugated with ethylene glycol are used, which provide a steric barrier outside the membrane. These liposomes remain in the blood for up to 100 times longer than conventional liposomes and can thus increase the biological efficacy of encapsulated agents (Lasic and Papahadjopoulos 1995). Coating liposomes with polysaccharides can also improve the stability of liposomes against enzymatic hydrolysis and disruption of liposomal structure by bile salts (Dong and Rogers, 1991).

1.6 AB block Copolymers

Many current efforts towards the selective delivery of drugs rely principally on vehicles (e.g., soluble polymers, liposomes, viruses). It is known that appropriate vehicle systems may provide means to rational drug therapy for intractable diseases (e.g., cancer) and genetic disorders. Two principal biological constraints for the selective delivery of drugs by vehicles to extravascular sites are rapid uptake by the reticuloendothelial system (RES) and difficulty in passage through the capillary endothelium from the vascular system; these may be overcome by polymeric micelles (Kataoka *et al.*, 1993).

1.6.1 Structure of AB Block Copolymers

Polymeric micelles may be composed of AB block copolymers which have a simple core/shell structure; the core consists of the hydrophobic segments and the corona region of hydrophilic segments. As the result of this amphiphilic property the AB type block copolymer can form a micellar structure. The hydrophobic segment forms the hydrophobic core of the micelle, while the hydrophilic segment surrounds this core as a hydrated outer shell (Kataoka *et al.*, 1993, Yokoyama *et al.* 1993). Such micellar structures may be considered as simple models for viruses and therefore, may demonstrate similar features which may be utilized for the effective presentation of drugs (e.g., size, *in vivo* persistence). The approach of tailoring block copolymer micelles as drug vehicles were initiated by Ringsdorf *et al.* along with the idea of mimicking lipoprotein structure (Pratten *et al.* 1985). They prepared an AB block copolymer in which the A-block is hydrophilic polyethylene oxide, and the B-block consists of biodegradable poly(L-Lysine) with a considerable substitution of the amino groups. Aforementioned examples of amphiphilic block copolymers have a hydrophobic moiety with a considerably flexible or mobile nature, which may form a core in a liquid state. However, it has become clear that amphiphilic block copolymers with glassy hydrophobic segments form a considerably stable structure of multimolecular micelles (Kwon *et al.* 1993a).

1.6.2 Preparation of Micelle-Forming Polymers

The syntheses of AB block copolymers which can form micelles are largely based on classic anionic polymerization techniques (Kataoka *et al.*, 1993, Kubo *et al.* 1995). Most block copolymers formed in such a manner, while relevant for the basic study of micellar phenomena, are composed of aliphatic backbones which are not biodegradable and usually not amenable for application *in vivo.* Recently much interest has focused on the development of polymeric micelles formed in aqueous systems which can be applied for *in vivo* studies. AB block copolymers, composed of polyethyleneglycol (PEG) and polyaminoacids, have been designed based on the rationale that PEG expresses low interaction with proteins and cells and has low toxicity. The hydrophobic polyaminoacid block would provide a biodegradable component as well as the required amphiphilicity for micellization. In principle, a systematic alteration in the structure of PEG-poly(amino acid) block copolymers through polymerization of varying amino acids or through side chain modifications, is attainable. They have apparent thermodynamic stability, and ability to deliver drugs selectively *in vivo* systems.

1.6.3 Advantages of AB Block Copolymers

Block copolymer micelles are promising drug vehicles, mimicking the natural carrier-systems with supramolecular structures (i.e., lipoproteins and viruses). Advantageous features of this delivery system can be summarized as: (1) formation of environmentally separated microcontainer of compounds through supramolecular assemblage, (2) installation of piloting moiety on the surface, (3) better duration in the biological compartment and (4) programmable stability (Kataoka *et al.*, 1993, Kwon *et al.* 1993b). The block copolymer micelles have a solubilizing effect typical of biologically active substances with a hydrophobic nature and with an advantage of less toxicity. These systems might be useful as a pharmaceutical adjuvant (Kataoka *et al.*, 1993, Hagan *et al.* 1995, Kubo *et al.* 1995).

There are few examples of block copolymers which are soluble in water and form micelles. Kabanov *et al.* (1992) used micellar drug delivery systems based on poloxamers for delivery of haloperidol, whereas Gref *et al.* (1994) loaded lidocaine in PLGA nanoparticles grafted with PEG. Adriamycin, a hydrophobic anticancer drug, has been conjugated with polyethylene oxide-polyaspartic acid block copolymers composed of various lengths of each block copolymer segment ranging from 1000 to 12000 in molecular weight and from 10 to 80 units, respectively. Furthermore, these conjugates have been found to form micellar structures with a hydrophobic inner core and a hydrophilic outer shell (Yokoyama *et al.* 1992). This micellar architecture may be utilized for effective drug targeting.

1.6.4 AB-Type Block Copolymers and Change in Physicopharmaceutical Properties of Drugs

An AB type block copolymer, composed of hydrophilic and hydrophobic components, can form a micellar structure as a result of its amphiphilic property. The hydrophobic segment forms the hydrophobic core of the micelle, while the hydrophilic segment surrounds this core as a hydrated outer shell. With a core-shell structure, polymeric micelles may maintain their water solubility by inhibiting intermicellar aggregation of the hydrophobic cores irrespective of high hydrophobicity of the inner cores. Drugs may be physically trapped or incorporated within polymeric micelles (Kataoka *et al.*, 1993, Kwon *et al.* 1993a, Yokoyama *et al.* 1992, Kubo *et al.* 1995). Indeed, a host of hydrophobic solutes has been solubilized in the interior of polymeric micelles. Also a hydrophobic drug may be covalently coupled to the polyaminoacids segment of an AB block copolymer with subsequent micellar formation and retention of high solubility, and stability. Furthermore, these loaded drugs may be released in *in vivo* or *in vitro* systems because of the dilution and destruction of micelles or enzymatic degradation of poly amino acid segments of the core.

The functions which are required for drug carriers can be shared by the structurally separated segments of the block copolymer. The outer shell is responsible for interactions with the biocomponents such as proteins and cells. These interactions may determine pharmacokinetic behaviour and biodistribution of drugs, therefore, *in vivo* delivery of drugs may be controlled by the outer shell independent of the inner core of the micelle which expresses pharmacological activities. This heterogeneous structure is more favourable to construct highly functionalized carrier systems than the conventional polymeric carrier systems.

1.7 Rationale of The Study

Taxol is one of the most interesting new anticancer drugs to enter the clinical trial in many years. This unique natural product with its novel mechanism of action and impressive clinical activity is a complex diterpenoid with particular structure features required for its activity. Taxol is under investigation as an antineoplastic agent in phase I, II and III human clinical trails. Target tumors include a variety of human cancers such as ovarian, breast, lung and colon as well as melanoma and lymphoma. Problems, including a limited supply of this natural product and poor aqueous solubility and stability, have almost prevented taxol from entering the clinic widely.

Many efforts have been made to increase taxol's supply, aqueous solubility, and stability while retaining the antitumor activity through structural modification or pharmaceutical formulation. However, none has clinically replaced the initial taxol:cremophor formulation, a lipid emulsion vehicle that has been reported to cause life-threating anaphylactoid reactions in animals and humans. Therefore, the clinical application of taxol has often been limited by its side effects, which including hypersensitivity reactions due to the rate and route of administration (iv) of its formulation (Koeller and Dorr, 1994). It has been reported that this kind of hypersensitivity reaction is associated with all drugs which have cremophor as a formulation vehicle. Changing the route of administration and replacing cremophor decreases or eliminates the incident of hypersensitivity reactions (Howric *et al.* 1985 and Koeller and Dorr, 1994). Therefore, an increased interest in developing new formulations and selecting other administration routes of taxol has heightened the need to understand the basic physicochemical properties of taxol. The oral route of administration has been chosen as an alternative to the iv route in order to reduce the dose-limiting side effects of this remarkable anticancer drug.

Bulky molecular structures such as taxol usually have low absorption rates. Also by oral delivery a greater variety of vehicles and excipients are available, allowing reduction or elimination of cremophor in the formulation of taxol. Therefore, the oral delivery of taxol is a practical approach to reduce side effects of taxol. In addition, the oral route is the most convenient and the most widely used way for drug administration, and during the process of drug development, oral bioavailability of a compound is a critical factor for the future of that compound as a drug.

Different results have been reported for oral bioavailability of taxol, indicative of its poor oral absorption (Fujita H. *et al.*,1994, Sparreboom *et al.*, 1996). Low oral bioavailability has the consequences of more variable and poorly-controlled plasma concentrations and consequent drug activity. On the basis of the above, the possibility of improving oral bioavailability of taxol was explored. In order to fulfill the above task, it was required to find the possible causes of the poor oral bioavailability of taxol. Oral bioavailability is primarily influenced by a drug's solubility, dissolution, stability and absorption across the intestinal epithelium as well as preabsorption and first pass clearance effects. Figure 1-8 shows the possible fate of a drug administered orally in a liquid dosage form.

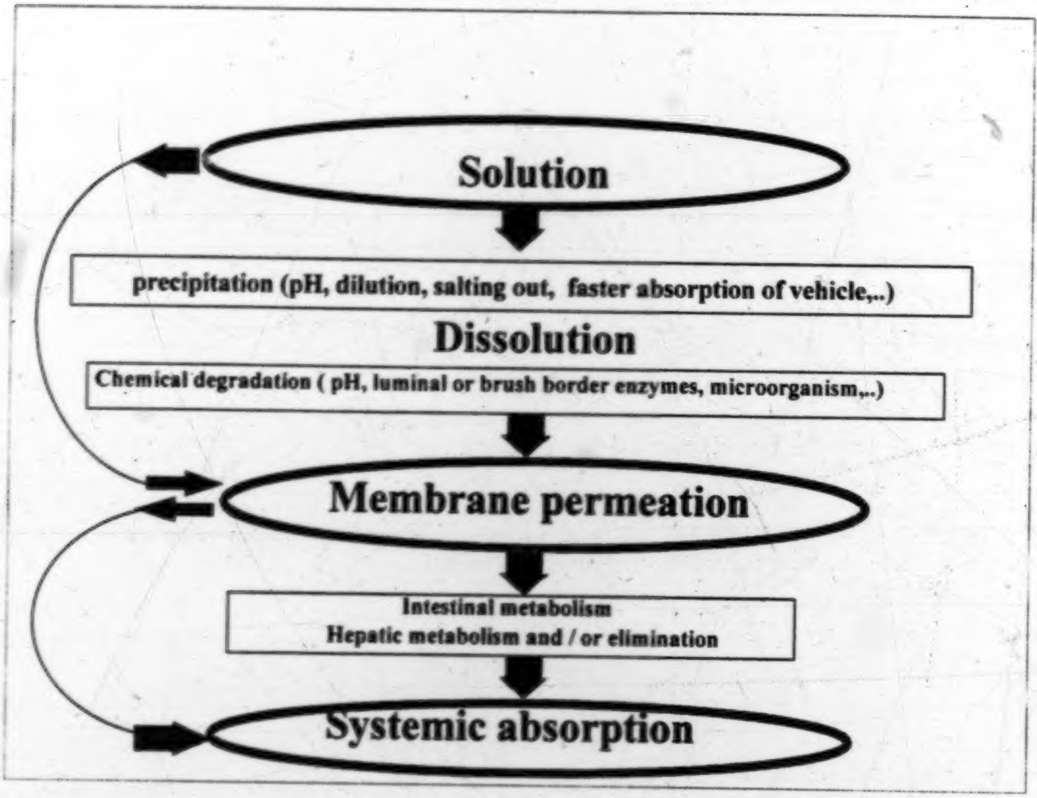
Solution
precipitation (pH, dilution, salting out, faster absorption of vehicle,..)
Dissolution
Chemical degradation (pH, luminal or brush border enzymes, microorganism,..)
Membrane permeation
Intestinal metabolism
Hepatic metabolism and / or elimination
Systemic absorption

Figure 1-8 **The possible fate of a drug administered orally in a liquid dosage form**

In order to possess good bioavailability for a drug, it must first be in a soluble form or produce enough soluble concentration inside the gastrointestinal tract for absorption. Secondly the soluble form of the drug must be stable enough to reach to the site of absorption and during the absorption process. Thirdly, during the absorption process the drug should not be metabolized by intestinal enzymes, and/or the drug should not undergo the first-pass metabolism. Usually this condition do not hold for all drugs. A solution of a drug may precipitate because of changes in pH, dilution, salting out, or fast degradation, metabolism or absorption of the vehicle. In that case the precipitation, the compound should have a good dissolution rate to produce enough concentration of the compound at the site of absorption. Membrane permeability can be an important barrier to the oral absorption of drugs and intestinal metabolism and hepatic clearance may eliminate the drug before reaching the systemic circulation. It has therefore, been proposed that the physicochemical parameters responsible for poor oral bioavailability of taxol, need to be investigated and the possibility of increasing oral bioavailability of taxol through the utilization of clinically established agents and methods, should be studied.

1.8 Hypotheses

Considering the foregoing background the following hypotheses needed to be tested:

1. The solubility, dissolution, and stability of taxol can be improved by cyclodextrins, nicotinamide, bile salts, liposomes and AB block copolymers.
2. Low permeability of taxol is the most important parameter responsible for its low oral bioavailability.
3. Complexation of taxol with nicotinamide and HPβCD change the partitioning of taxol into gut cell wall membrane.
4. Nicotinamide and HPβCD affect the cell toxicity of taxol.
5. Oral bioavailability of taxol improves in the presence of nicotinamide, HPβCD and NSAIDs

1.9 Objectives

1. Determine the solubility, stability, dissolution profiles of taxol in water as a function of temperature, pH, and the presence of different solubilizing agents.
2. Determine the permeability of taxol in everted rat gut as a function of formulation, dose, and the presence of solubilizing agents.

3. Determine the partitioning of taxol in immobilized artificial membrane in the presence of solubilizing agents.
4. Determine the cell toxicity of taxol in the presence of solubilizing agents.
5. Determine the bioavailability of taxol in the rat after oral and intraperitoneal administration.
6. Determine the bioavailability of taxol in rat after oral administration of various formulations.
7. Compare the effect of pretreatment with flurbiprofen on the oral bioavailability of taxol in the rat after oral administration in cremophor formulation.
8. Determine the relative GI toxicity of taxol in the rat after oral administration of taxol in cremophor, nicotinamide and hydroxypropyl-β-cyclodextrin formulations.

1.10 General References

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Chapter 2

2. Effect of pH, Temperature, and Different Solubilizing Agents on the Solubility of Taxol.

2.1 Introduction

Taxol, a complex diterpene isolated from the bark of Taxus brevifolia (*Pacific yew*), is currently considered the most exciting lead in cancer chemotherapy (Koeller and Dorr, 1994). Taxol possesses high cytotoxicity and strong antitumor activity. Significant activity against cisplatin refractory advanced ovarian cancer has been established and FDA has approved the clinical use of taxol since 1992. For other cancers such as breast cancer and lung cancer, taxol is currently in phase III clinical trials in the United States. However, a notable property of taxol is its poor solubility in aqueous media, which necessitates its administration in an oily vehicle, such as polyethoxylated castor oil containing 50% absolute ethanol (cremophor EL). However, cremophor EL is implicated in hypersensitivity reactions after infusion of taxol. Efforts have been made to synthesize water-soluble derivatives of taxol, however, these analogs have not been found clinically favorable over the taxol:cremophor formulation (Koeller and Dorr 1994).The aim of this study was to improve the solubility of taxol through the utilization of clinically-established solubilizing agents, such as nicotinamide, PEG, bile salts, and cyclodextrins.

2.2 Materials and Methods

2.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA, and used as supplied. The following cyclodextrins were obtained from Aldrich Chemical Company, Milwaukee, WI, USA, and were used without further purification: α-cyclodextrin (αCD), ß-cyclodextrin (ßCD), hydroxypropyl-ß-cyclodextrin (HPßCD, molecular weight=1380). Nicotinamide, PEG, and bile salts (50:50 sodium cholate:sodium deoxycholate) were supplied by Sigma Chemical Company, St. Louis, MO, USA. γ-cyclodextrin (γCD) was obtained from American Maize-Products company, Hammond, IN, USA. Water was deionized, Milli-Q (Millipore) purified. All other materials were reagent grade.

2.2.2 Solubility Measurements

Solubility studies were conducted by adding excess amounts of taxol to aqueous solutions containing various amounts of one of αCD, βCD, γCD, HPβCD, bile salts, and PEG and nicotinamide, in a 15-ml screw-capped vial and sonicated for 3 hrs in a bath sonicator (20% output, pulsed, 20° C using sonicator W-375 (Heat Systems-Ultrasonics, Inc.)). After equilibration in the dark at room temperature for 24 hrs, the resulting samples were centrifuged, filtered (Nalgene, 0.2 µm, 25-mm disposable syringe filter) and prepared for analysis. In addition the solubility of taxol was measured as a function of temperature (25, 37, 50, and 60° C) and pH values of 1.2, 2.5, 4.5, (citrate buffers, 0.1 molar) and 6.5, 7.4, and 8.0 (phosphate buffers, 0.1 molar). The effect of pH on the solubility of taxol was determined in 20% w/v aqueous γCD, HPβCD, and nicotinamide solutions respectively, at 25° C. The solubility of taxol was analyzed as a function of temperature, and the data treated according to the Van't Hoff and Hildebrand equations (Yu X., *et al.* 1994). All measurements were made in triplicate and averaged.

2.2.3 Method of Analysis of Samples

Samples were analyzed for taxol using a Waters HPLC system (Lc module 1) consisting of a model 600E pump, a model 715 auto-injector, and a model 486 UV detector, with a partisil ODS (phenomenex ® 100 mm, 4.6 mm ID, 5 µm particle size) column protected by a partisil ODS (phenomenex ® 30 mm, 4.6 mm ID, 5 µm particle size) guard column, using a mobile phase of 40% acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.0) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm. Samples of 1 ml plus 0.1 ml of internal standard (fenoprofen 3.5 µg/ml in 47% acetonitrile) were extracted with 3.5 ml of *t*-butyl methyl ether and vortex-mixed for 1 min. The mixture was then centrifuged for 10 min at 1100 g after which 3 ml of the organic layer were removed and evaporated to dryness under vacuum at low temperature.

The residue was reconstituted in 0.2 ml of 47% aqueous acetonitrile and injected (50 μl) into the HPLC system. Standard solutions of taxol were made when appropriate, extracted and injected before, during, and after a series of samples was injected.

2.2.4 DSC Studies

A Seiko SII (model SSC /5200) differential scanning calorimeter was used for all measurements. The transition temperature and transition energy were obtained simultaneously prior to the measuring the melting profile of high-purity indium. For each experiment, approximately 1mg of taxol was sealed in a hermetic aluminum pan, with a 1mg aluminum encapsulated pan as reference. The temperature program was run with a linear ramp of 10° C/min from 100 to 180° C and 2° C/min from 180 to 260° C. The cell was purged with nitrogen throughout the duration of the DSC run with a flow rate of 50 ml/min.

2.2.5 Statistical Evaluation

Statistical evaluations were performed on all data using ANOVA and One-way ANOVA unless otherwise stated. Where a significant F-value was obtained, the Student-Newman-Keuls method was used for pairwise multiple comparisons, the level of significance chosen for all statistical analyses being $\alpha = 0.05$. The results were expressed as mean ± standard error of the mean (SEM).

2.3 Results and Discussion

The maximum solubility of taxol in water was found to be 320 μg/L at 25 °C. The solubility of taxol in water was enhanced substantially by increasing the temperature within the examined range of 25-60° C (Figure 2-1). Increasing the temperature from 25 to 37° C resulted in a 2-fold increase in solubility of taxol.

Both the Van't Hoff and Hildebrand equations (Yu, X *et al.* 1994) were used to analyze temperature dependence of taxol intrinsic solubility. Regression analysis of the data in Van't Hoff and Hildebrand plots yielded the following respective equations:

$\ln X = (-5.48 \times 10^3)/T + 0.0458, r = 0.985$ Van't Hoff plot

$\ln X = (-17.87 \ln T - 117.48), r= 0.983$ Hildebrand plot

Where X is the mole fraction solubility of taxol and T is the applied temperature. The heat of fusion (ΔH_m) determined from the slopes of the respective plots and from the DSC experiments are shown in Table 2-1. Compared to the DSC result (Figure 2-2) the calculated ΔH_m values show that the Hildebrand method and the Van't Hoff equation gave underestimated results. However, from the absolute values of the relative errors, the Hildebrand plot produces a better estimation of the heat of fusion of taxol and, hence, its intrinsic solubility.

The effect of pH on the solubility of taxol is shown in (Figure 2-3). Taxol solubility was essentially pH-independent at 25° C. It appears from the data that taxol contains no groups that are ionizable in an acceptable pH range and therefore, pH adjustment dose not produce a significant increase in the solubility of taxol.

2.3.1 Cyclodextrin Systems

By virtue of an ability to form inclusion complexes with many drugs, cyclodextrins can substantially increase the aqueous solubility of pharmaceuticals (Brewster, 1989, Uekama 1985 1987, and 1995, Torres-Labandeira JJ 1991, Cserhati T., 1994 and 1995, Vincieri, 1995, Masuda *et al.* 1984, Tarimci and Celebi, 1988, Zecchi *et al.* 1988, and Loftsson *et al.* 1993). Cyclodextrins are classified by the number of α-1,4-linked glucose units which occur in their molecular structure. αCD has six such units, βCD has seven and γCD, has eight. In these compounds, the C-1 chain conformations of the glucose monomers arrange the molecule in a cone-like structure in which the hydroxy groups are oriented towards the exterior (Szejtii, 1982 and 1994). The narrower end of the cone contains the primary hydroxy functionality while the wider end has the secondary hydroxyl groups (Figure 1-6). This arrangement makes the cyclodextrin exterior predominantly hydrophilic. The secondary hydroxyl groups are able to interact via hydrogen bonding to stabilize the crystalline lattice. This reduces the solubility of cyclodextrins to a large extent, especially that of βCD, in water. The interior of the cyclodextrin cone is predominantly hydrophobic due to assembly of the skeletal hydrocarbon chains. Hence, the interior of the cone structure of cyclodextrin represents a non-polar environment suitable for solubilizing poorly water-soluble compounds (Brewster, 1989, Uekama 1981, 1987). Generally, cyclodextrins are water-soluble compounds which can form reversible complexes with poorly water-soluble molecules such as taxol resulting in a soluble molecular inclusion complex. Due to its large molecular weight (854 d), taxol does not fit entirely into the cyclodextrin cavity. Nevertheless, a partial inclusion (e. g., side chains) seems to be sufficient to increase aqueous solubility of drugs (Brewster, 1989).

Characteristics of taxol inclusion complexes formed with cyclodextrins and taxol were determined by examining the effect of solubilizer concentration on taxol solubility. The resulting phase-solubility profile of taxol in αCD, βCD, γCD, and HPβCD is shown in figures 2-4 to 2-7. According to the definition of Higuchi and Connors, (1965), the two main types of solubility profiles, A and B, can be seen for taxol. A-type curves indicate the formation of soluble inclusion complexes, while B-type relationships indicate the formation of complexes with limited solubility. Each type is further subdivided. A plot of concentration of solubilized taxol (M) versus the HPβCD concentration (M) is linear, while taxol solubility is not a linear function of γCD concentration. Therefore, A_L-type and A_P-type systems are expected for inclusion complexes of HPβCD and γCD with taxol, respectively. Complexes of taxol with α- and β-cyclodextrin have low solubility and thus a B_I-type curve is generated. For A-type systems, the initial linear portion of the curve was used in examining the efficacy of complexation. The steeper the slope, the better the complexation and at a ratio of 1:1, one mole of cyclodextrin complexes with one mole of drug, the process is considered 100% efficient. The solubility constant (K) for the 1:1 complex of taxol and HPβCD was estimated from the following equation:

$$K_{1:1} = \text{Slope}/[S_o(1\text{-Slope})]$$

For the initial linear portion of an A_L-curve, S_o refers to the solubility of taxol in the absence of cyclodextrin. As shown in Figure 2-4, the solubility of taxol increased linearly as a function of HPβCD concentration, ranging from 0.5 to 20% w/v. The slope of the phase solubility curve gave a value of 1×10^{-4} indicating an efficient HPβCD-taxol interaction. The apparent stability constant for the HPβCD:taxol (1:1) complex was estimated to be 267 M^{-1}, indicating that a relatively stable complex was formed. Taxol solubility was not a linear function of γCD concentration suggesting the formation of higher-order complexes. For γCD with an A_P-type relationship, $K_{1:2}$ constants were obtained from the phase solubility curve which can be fitted to a quadratic equation from:

$$S_t = S_o + K_{1:2}\,S_o(CD) + K_{1:1}\,K_{1:2}\,S_o(CD)^2$$

where S_t is the total taxol solubilized and CD is the γ-cyclodextrin concentration. Some of the calculated data are shown in Table 2-2. Usually the stability constants of cyclodextrins with drugs are in the order of 10-10,000 M^{-1}. Very low stability constants indicate that the drugs will not appear to form complexes very readily unless relatively high concentrations of cyclodextrin are present. No relationship has yet been established between the stability constants measured and aqueous solubilization enhancement by cyclodextrins. Since the stability of the complex is more dependent on the number of the stabilizing interactions rather than on the strength of each interaction (which are absolutely quite small), rapid changes in the concentration of the drug, cyclodextrin, and water can cause rapid shifts in equilibrium. Simply by diluting the equilibrium mixture, one can shift the equilibrium to favor the availability of free drug. The pH exerted a profound effect on taxol solubility in 20% w/v solutions of, γCD, and HPβCD (Figures 2-8 and 2-9). At pH 1.2 the solubility of taxol in 20% HPβCD, was significantly lower than in solutions with pH higher than 1.2. However no significant difference between solubility of taxol in 20% HPβCD solutions of pH between 2.5 to 8.0 was observed.

Maximum solubility of taxol in 20% w/v γCD was attained at pH 4.5, however, in all those buffered solutions of, 20% w/v γCD, and HPβCD, the solubility of taxol was significantly lower than in solutions of γCD and HPβCD in water. At pH 4.5 solubility of taxol in solutions of, 20% w/v γCD, or HPβCD were reduced by 50% and 20%, respectively, as compared to unbuffered solutions of γCD and HPβCD (20% w/v), possibly due to salting out of taxol by the buffer components or the effect of buffer component on the inclusion complexation of cyclodextrins and taxol.

Among the cyclodextrins significant solubilization of taxol was observed in γCD and HPβCD solutions but not in αCD and βCD solutions. It appears that only some lipophilic part of taxol can fit into the lipophilic part of the cyclodextrin structure, rather than the entire molecule. Therefore, the larger cavity of HPβCD with greater lipophilicity inside and greater hydrophilicity outside yielded the largest solubility increase of taxol.

2.3.2 The Nicotinamide System

Nicotinamide (3-pyridinecarboxamide) is a water-soluble, nontoxic B vitamin (vitamin B_3). Through complexation, nicotinamide has been shown to enhance the aqueous solubility of many drugs (Hata *et al.* 1970, Fawzi *et al.* 1980, Truelove, *et al.* 1984, Hamza and Parrots 1985, Rasool *et al.* 1990, Hussain *et al.*, 1992). The exact mechanism by which this compound forms complexes is not entirely clear. The solubility enhancement of certain heteroaromatic drug molecules, steroids, menadion, and acetaminophen by complex formation with nicotinamide have been explained by the concepts of hydrotropy or a *Pi*-donor *Pi*-acceptor interaction (Fawzi *et al.* 1980, Hamza and Parrots 1985, Rasool *et al.* 1990).

Although taxol has a very different chemical structure and physical properties compared to previously studied drugs (Hata *et al.* 1970, Fawzi *et al.* 1980, Truelove, *et al.* 1984, Hamza and Parrots 1985, Rasool *et al.* 1990, Hussain *et al.*, 1992), almost all of them, including taxol, have a carbonyl group and a *Pi*-conjugated system in their molecular structures. Therefore, by extrapolation, the nicotinamide:taxol interaction can be assumed to occur by a similar mechanism. Figure 2-10 shows that the solubility of taxol was enhanced with increasing nicotinamide concentration (0.5 to 20% w/v) up to 95-fold, compared to water (0.32 mg/L). It is evident that the solubility of taxol in nicotinamide was enhanced significantly as a function of nicotinamide concentration in a nonlinear fashion. For nicotinamide with an A_P-type relationship, $K_{1:1}$ and $K_{1:2}$ constants were obtained from the phase solubility curve and the solubility of taxol was expressed in the form of a quadratic equation as follows:

$$S_t = S_o + K_{1:2} S_o (ND) + K_{1:1} K_{1:2} S_o (ND)^2$$

where S_t is the total taxol solubilized and ND is the nicotinamide concentration. Results derived from calculations are shown in Table 2-2. In order to explain the phase solubility diagrams mathematically, it was assumed that a linear increase in solubility may be attributed to the formation of 1:1 complexes, whereas a nonlinear increases can be attributed to the formation of higher-order complexes. It is speculated that these higher-order complexes are formed as the result of different types of interactions. One such interaction may involve the formation of dimers or n-mers of the nicotinamide and drug. Results of dielectric constant measurements have shown that, over the concentration range of 20-40% (w/v), nicotinamide has only a very small effect on the dielectric constant of water (78.5). The dielectric constant of nicotinamide solutions in water up to 40% w/v has been shown to be only a few tenths below that of pure water (Rasool *et al.* 1990). This suggests that a change in the dielectric constant of water was not involved in the nonlinear solubility increase of taxol. The results of surface tension measurements show that the surface tension of 20% (w/v) nicotinamide in water was only 15% lower than that of pure water (Figure 2-11). This is consistent with the findings of Rasool *et al.* (1990), who reported the extent of self-association of nicotinamide was very small. This suggests that self-association of nicotinamide is not a major factor involved in the nonlinear increase of taxol solubility in nicotinamide solutions. If it is assumed that the enhancement of taxol solubility in nicotinamide solution is mainly due to *Pi*-system interaction, then the existence of plane-to-plane stacking of taxol and nicotinamide molecules is probable.

The solubility of taxol in nicotinamide solutions was also pH-dependent. At pH 4.5 a maximum taxol solubility of 59 mg/L occurred in 20% (w/v) nicotinamide solution (which was significantly different from other pH), and the lowest solubility was observed over the range pH 1.2-2.5 (Figure 2-12). Nicotinamide is a weak base and is almost neutral in reaction in water and it forms acid salts. The stability of nicotinamide is greatest between pH 4.5 and 6.0 and in strongly acid or strongly alkaline solution hydrolysis does occur (Roth *et al.* 1994). Hence, in acidic solutions decomposition of nicotinamide may be partly responsible for the low solubilities observed. However, it is most likely that the pH affects the stability of nicotinamide and the nicotinamide:taxol complex in a negative fashion and the optimum stability condition can be achieved at pH 4.5. This finding can support the suggestion that the structure of nicotinamide and specially the aromaticity (*Pi*-system) of the pyridine ring is the main contributing factor in the nonlinear solubility behavior of different compounds in the presence of nicotinamide (Rasool *et al.* 1990).

In order to determine if pH altered the extent of self-association of nicotinamide, and thereby its solubilizing effect, the surface tensions of solutions of nicotinamide (0-20 %w/v) were determined as a function of pH (1.2, 4.5, 6.8, and 8.0) using the Wilhelmy balance technique (Figure 2-13). At pH 1.2 slight variations of surface tension were observed at different nicotinamide concentrations. The surface tensions at pH 4.5 decreased initially (up to 2.5%w/v nicotinamide concentration) then remained fairly constant between 5.0 to 20 % w/v nicotinamide concentration. At pH 8 the initial decrease in surface tension was more gradual but also became relatively constant from 10-20% w/v nicotinamide.

These results suggest that although the self-association of nicotinamide is weak but, significant changes do occur as a function of pH which could influence the solubilizing effect of nicotinamide. At pH 4.5 the self-association of nicotinamide molecules appears to be the most stable and most accommodating yielding, in turn, solubilization of taxol. At pH 1.2 nicotinamide has the lowest stability and self-association and therefore, the lowest solubilizing effect was observed. The above results indicate that the solubilization effect of nicotinamide is controlled with the level and stability of self-association of nicotinamide molecule.

2.3.3 Bile Salt Effects

Bile salts are the major products of cholesterol metabolism and, biologically, the most important detergent-like molecules. The principal bile salts in humans are cholic acid and chenodeoxycholic acid. These amphipathic molecules possess hydrophilic (two to four polar groups) and hydrophobic segments (cholesterol system) in their structures which enable them to display a unique behavior in water. In aqueous solutions these molecules come together to form aggregates which usually are denoted as micelles. The nonpolar parts are located in the core region of the micelle, whereas the polar groups interact with water around the periphery of the micelle. The physicochemical properties of bile salts are similar to those of typical colloidal surfactants, including their ability to solubilize water-insoluble substances above their critical micelle concentrations (CMC). In micellar solubilization the incorporation of non-polar compound into micelles (that is, aggregates composed of monomers of the surfactant), occurs above the CMC of the surfactant (Bates *et al.* 1966 and Rosoff *et al.* 1980). Bile salts have been reported to increase the apparent solubility of many poorly water-soluble drugs such as diazepam, griseofulvin, NSAIDs, 1,8-dinitropyrene, triamcinolone, betamethasone, and dexamethasone (Bates *et al.* 1966, Rosoff *et al.* 1980, Miyazaki *et al.* 1981, Coello *et al.* 1996). The CMC of bile salts is (13-16 mM or higher).

Figure 2-14 shows the effect of bile salts (50:50 sodium cholate:sodium deoxycholate) on the solubility of taxol. The solubility of taxol increased with increasing bile salts concentration from 0.5 to 20% w/v and solubility increased to 45-fold, compared to water (0.32 mg/L) at 20% w/v. The bile salts dependence of taxol solubilization is nonlinear suggesting that the extent of taxol solubilization is a function of the micelle concentration, as well as shape and aggregation.

2.3.4 Polyethylene Glycol and Ethanol Effects

Organic cosolvents are among the most powerful solubilizing agents for a large number of lipophilic drugs (Li and Yalkowsky 1994). Addition of cosolvents to water can lower the polarity of the water and thereby increase the solubility of poor water-soluble drugs. Figures 2-15 and 2-16 show the effect of PEG and ethanol on the solubility of taxol, respectively. The solubility of taxol was enhanced as a function of PEG or ethanol concentration increasing taxol solubility to 498- fold and 651-fold in 50% v/v in PEG and ethanolic solutions, respectively, as compared to water (0.32 mg/L). It is apparent that PEG and ethanol enhance the solubility of taxol in a nonlinear fashion as a function of co-solvent concentration. Being less polar than water, addition of PEG and ethanol to water decreased the polarity of water to a more favorable value. The rule of polarity adjustment on the solubility of taxol was more pronounced with ethanol than in PEG solutions as expected.

2.4 Conclusions

The influence of pH, temperature, PEG, nicotinamide, bile salts and four different cyclodextrins on the solubility of taxol were investigated. This study showed that solubility of taxol in water is not pH-dependent and hydroxypropyl-β-cyclodextrin (20% w/v), nicotinamide (20% w/v), bile salts (20% w/v), ethanol (50% v/v) PEG 400 (50% v/v) significantly increased the solubility of taxol from 0.32 μg/ml to 16, 59, 14, 208, and 160 μg/ml. Further studies are required to investigate the possible changes in other physicopharmaceutical and pharmacokinetic disposition of taxol with these solubilizing agents and thereby its therapeutic effects.

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Table 2-1 Heat of fusion for taxol from solubility and DSC studies.

	Van't Hoff Eq.	Hildebrand Eq.	DSC
ΔH_m (Kcal/mol)	10.89	16.56[a]	19.17
Relative Error[b]	76.0%	15.8%	0%

[a] Onset temperature of taxol from DSC study is 206 °C.

[b] Relative error, compared to DSC result.

Table 2-2 Summary of complexation constants of taxol with γCD, HPβCD, and nicotinamide, based on Higuchi and Connors method.

	Slope	Intercept	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-2})
HPβCD	1×10^{-4}	1×10^{-7}	266.0	-
γCD	3×10^{-5}	2×10^{-6}	8.06	800
Nicotinamide	1×10^{-5}	2×10^{-6}	5.3	26.6

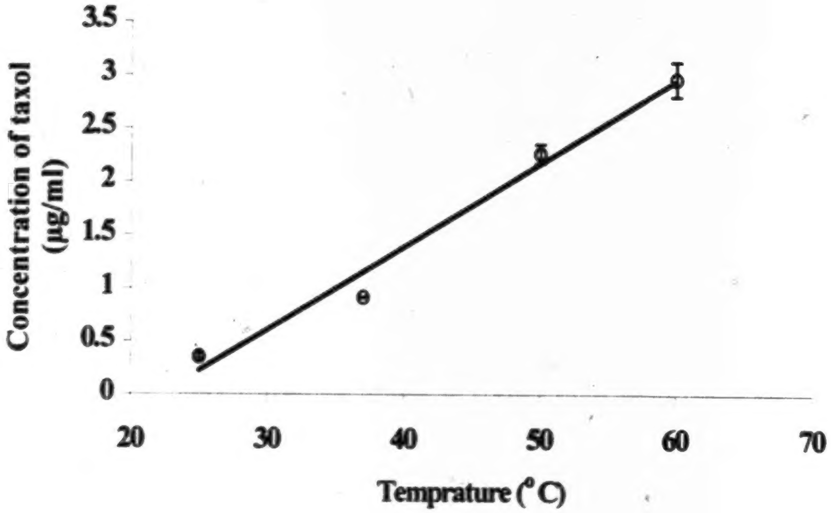

Figure 2-1 **Effect of temperature on the solubility of taxol in water. Data are presented as the mean ±SEM (n=3).**

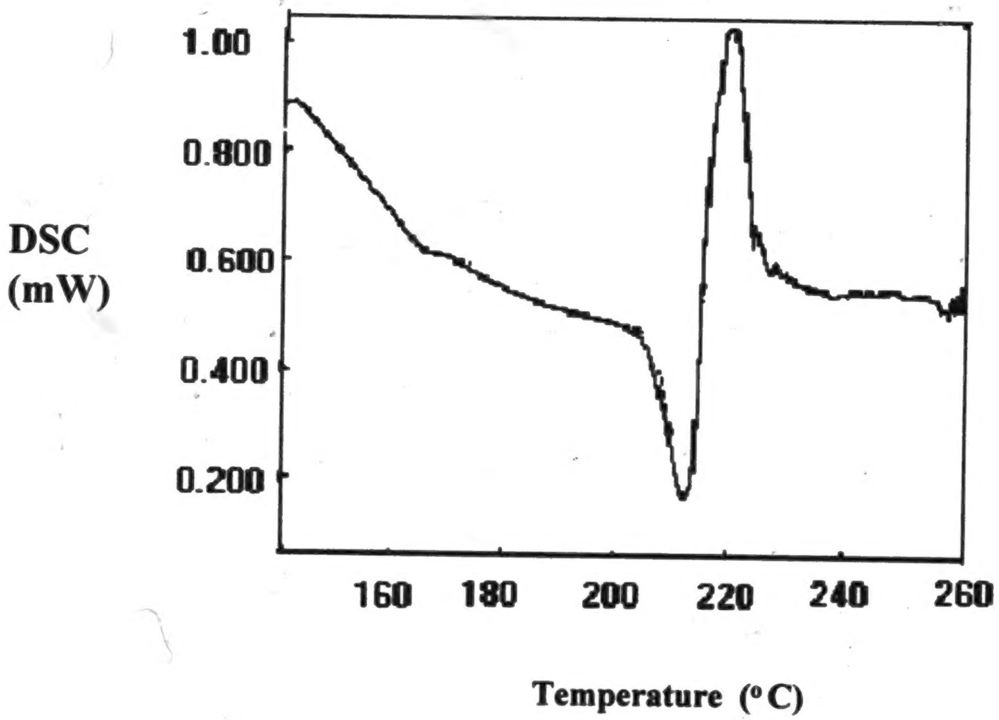

Figure 2-2 DSC thermogram of taxol. The temperature program was run with a linear ramp of 10° C/min from 100 to 180° C and 2° C/min from 180 to 260° C.

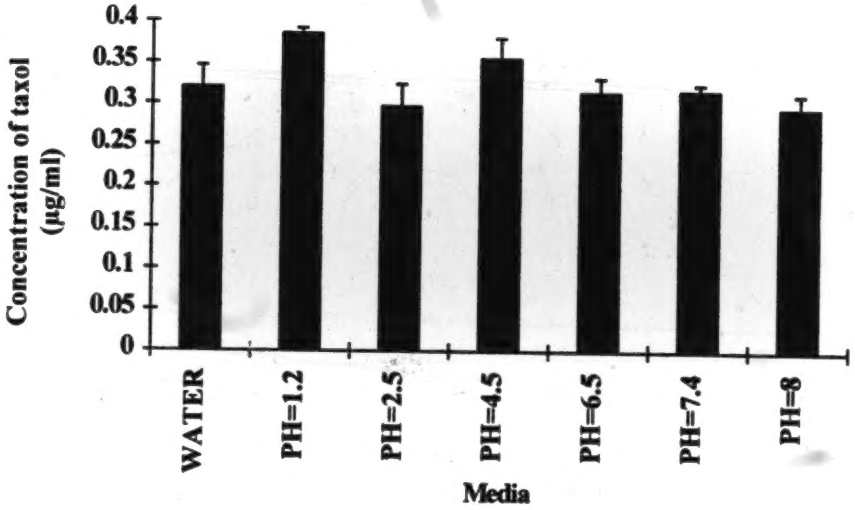

Figure 2-3 The effect of pH on the solubility of taxol in water. Data are presented as the mean ±SEM (n=3).

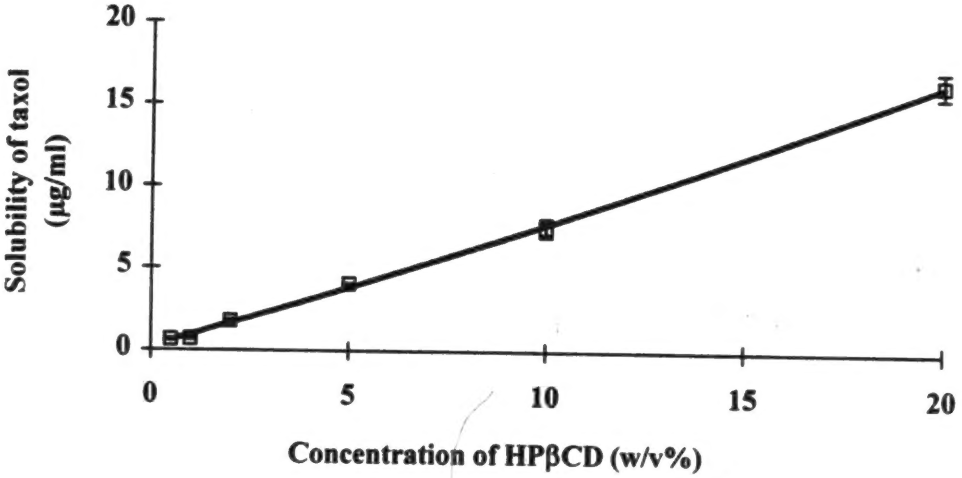

Figure 2-4 Phase-solubility diagram of taxol in water in the presence of HPβCD. Data are presented as the mean ±SEM (n=3).

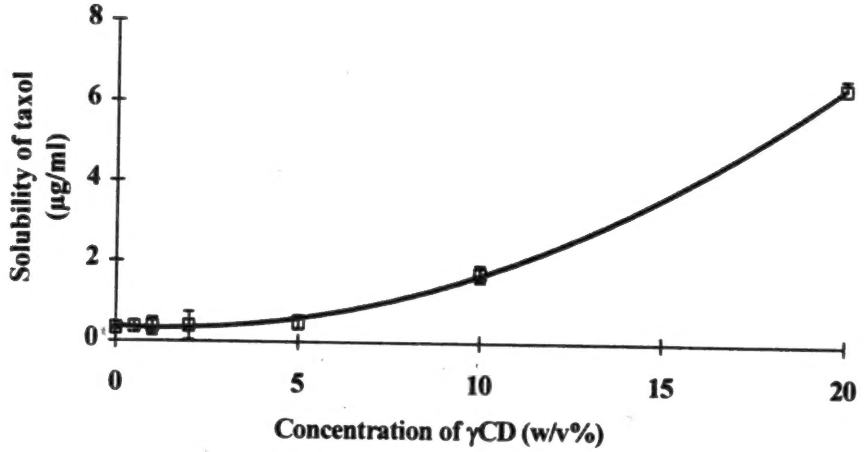

Figure 2-5 Phase-solubility diagram of taxol in water in the presence of γCD. Data are presented as the mean ±SEM (n=3). Solid line is the best fit through the data points (polynomial).

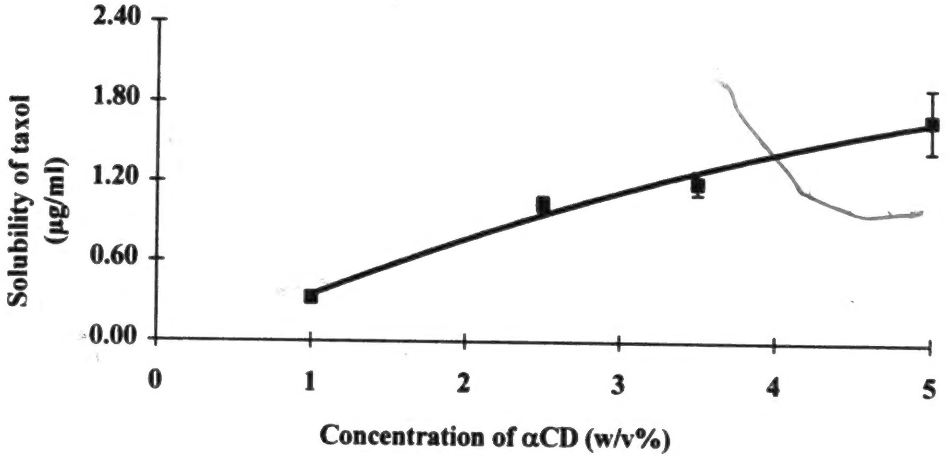

Figure 2-6 Phase-solubility diagram of taxol in water in the presence of αCD. Data are presented as the mean ±SEM (n=3). Solid line is the best fit through the data points (polynomial).

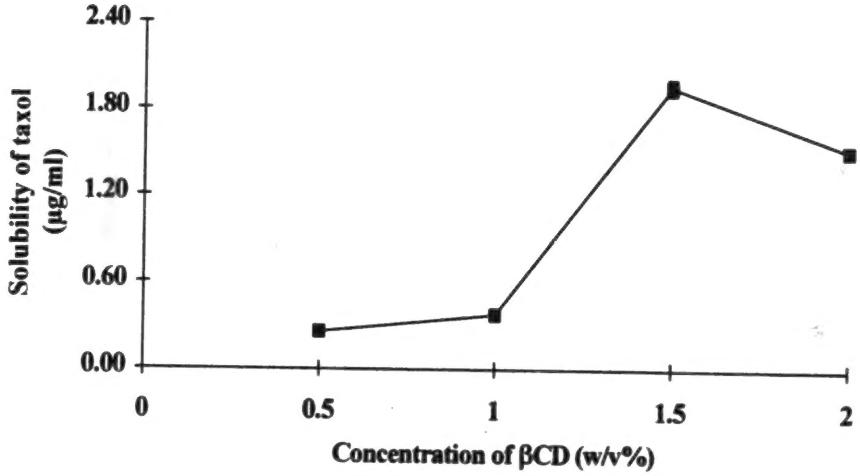

Figure 2-7 Phase-solubility diagram of taxol in water in the presence of βCD. Data are presented as the mean ±SEM (n=3).

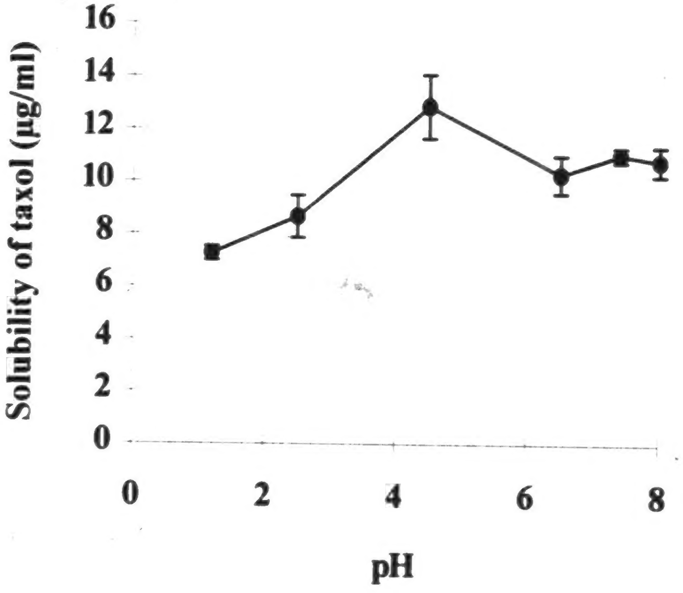

Figure 2-8 Effect of pH on solubilization of taxol in 20% w/v HPβCD solution. Data are presented as the mean ±SEM (n=3).

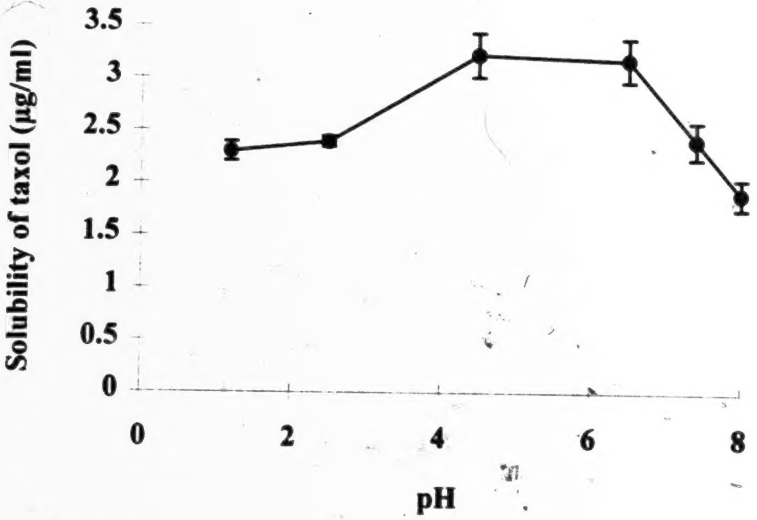

Figure 2-9 Effect of pH on solubilization of taxol in 20% w/v γCD solution. Data are presented as the mean ±SEM (n=3).

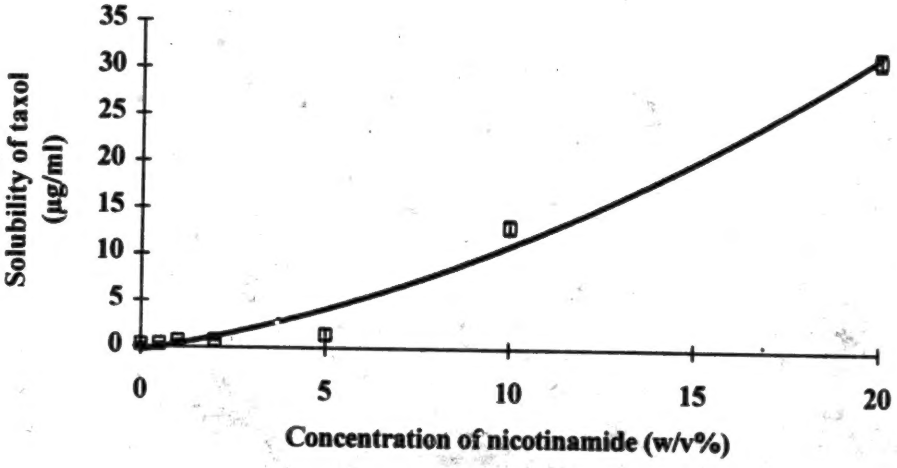

Figure 2-10 Phase-solubility diagram of taxol in water in the presence of nicotinamide. Data are presented as the mean ±SEM (n=3). Solid line is the best fit through the data points (polynomial).

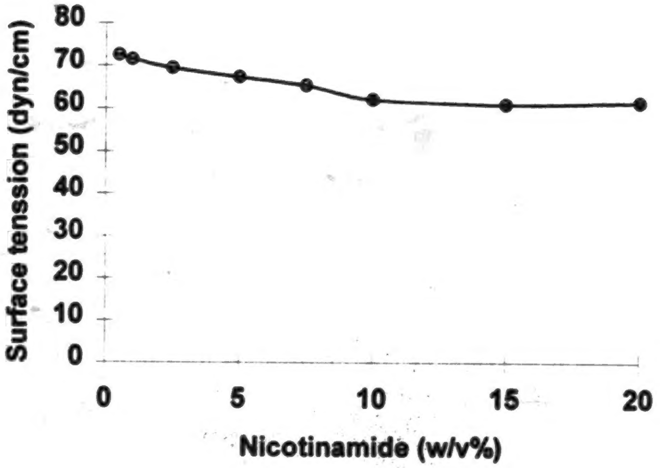

Figure 2-11 Apparent surface activity of nicotinamide in water at room temperature. Data are presented as the mean ±SEM (n=3).

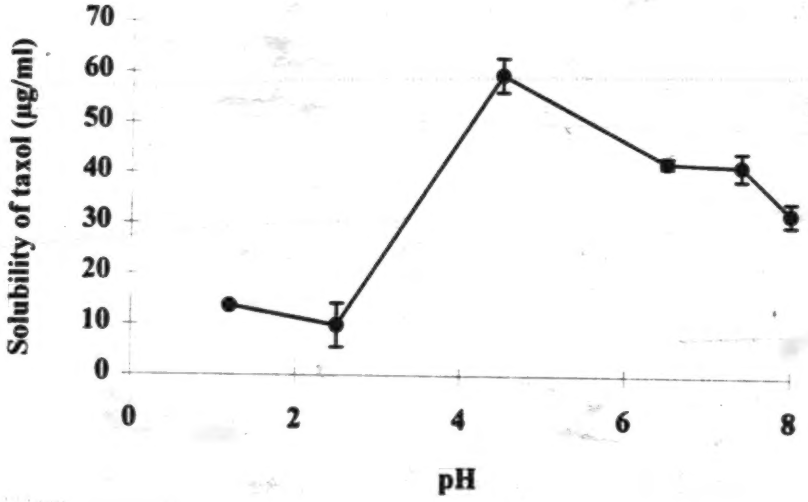

Figure 2-12 Effect of pH on solubilization of taxol in nicotinamide solution (20% w/v). Data are presented as the mean ±SEM (n=3).

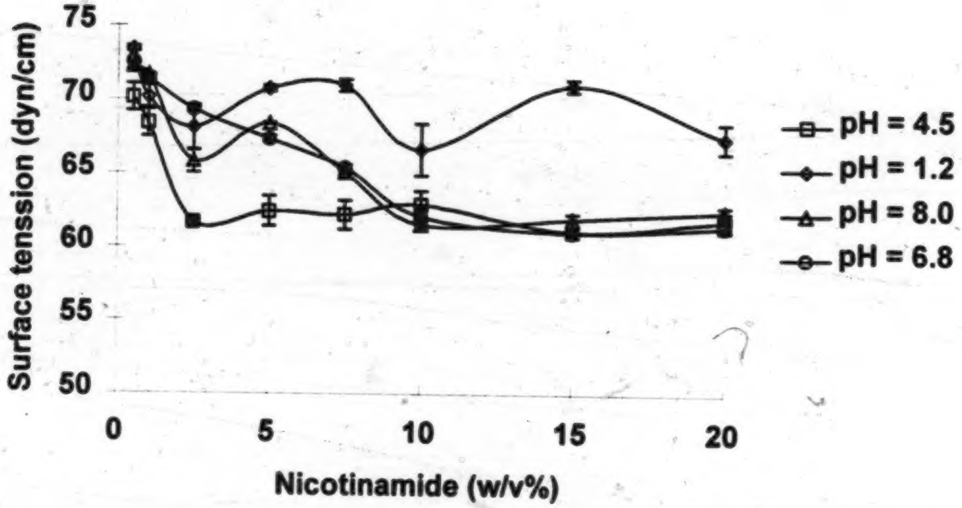

Figure 2-13 Effect of pH on surface tension of nicotinamide in water. Data are presented as the mean ±SEM (n=3).

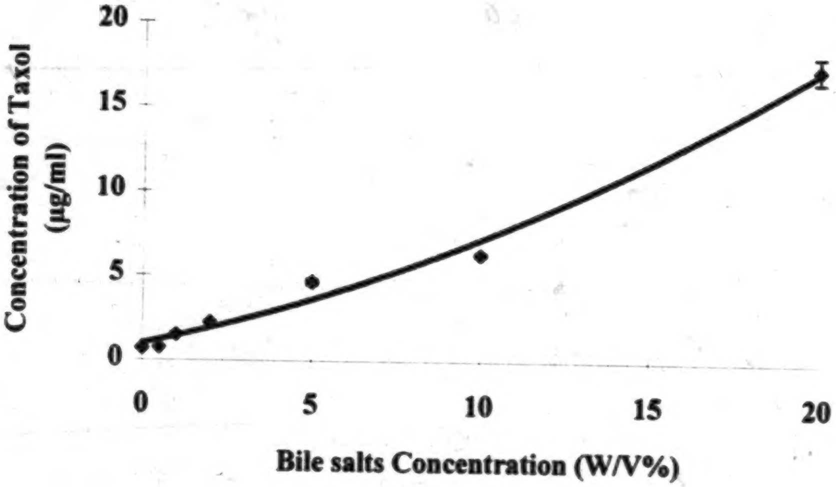

Figure 2-14 Phase-solubility diagram of taxol in water in the presence of bile salts. Data are presented as the mean ±SEM (n=3). Solid line is the best fit through the data points (polynomial).

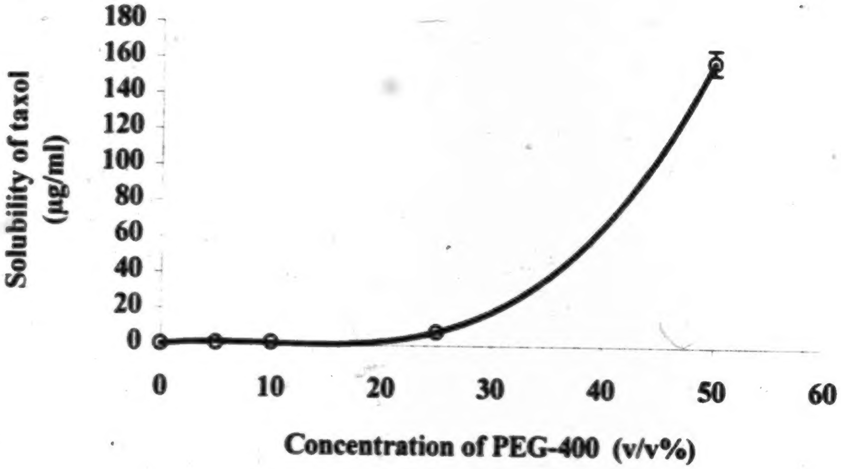

Figure 2-15 Phase-solubility diagram of taxol in water in the presence of PEG 400. Data are presented as the mean ±SEM (n=3). Solid line is the best fit through the data points (polynomial).

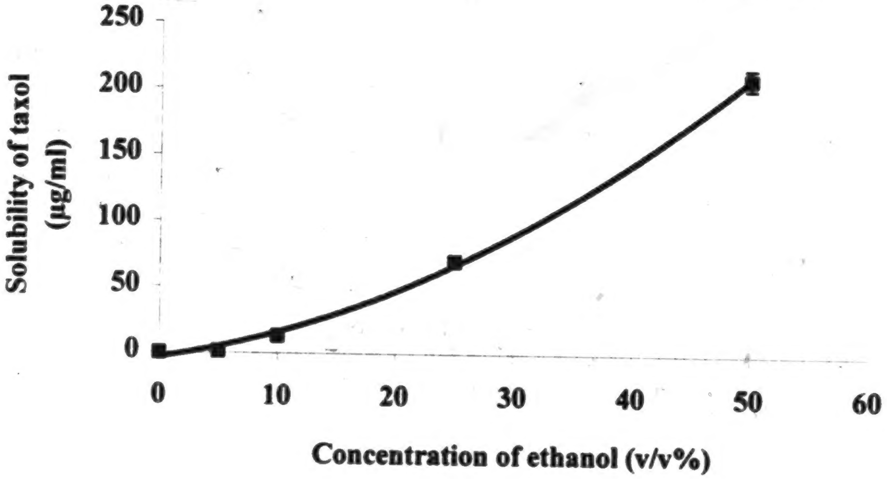

Figure 2-16 Phase-solubility diagram of taxol in water in the presence of ethanol. Data are presented as the mean ±SEM (n=3). Solid line is the best fit through the data points (polynomial).

Chapter 3

3. PEG-PBLA Nanoparticles and DPPC:CHOL Liposomes as Potential Solubilizing and Stabilizing Systems for Delivery of Taxol.

3.1 Introduction

Poor aqueous solubility has limited the clinical application of taxol. Many methods have been attempted to improve aqueous solubility of taxol while retaining its antitumor activity, but none has been successful in replacing the clinical utilization of taxol:cremophor formulation. Cremophor is a polyoxyethylated caster oil derivative which has been reported to cause life-threating anaphylactoid reactions in animals and humans. Hence, other approaches of increasing the amount of taxol in solution beyond its low water solubility are needed. Two microparticalable systems have been investigated for their molecule inclusion potential via association with either AB block copolymers, or liposomes

3.1.1 AB Block Copolymers

Polymeric micelles are promising drug vehicles which are able to mimic the natural carrier-systems such as lipoproteins and viruses (Pratten *et al.* 1985, Kataoka *et al.* 1992, Kwon *et al.* 1993, Kabanov *et al.* 1994, Gref R., *et al.* 1994, Kubo *et al.* 1995, Hagan *et al.* 1995). Polymeric micelles may be composed of AB block copolymers which have a simple core/shell structure; the core consisting of the hydrophobic segments and the corona region of hydrophilic segments (Figure 3-1). As a result of these amphiphilic properties an AB type block copolymer can form a micellar structure. Such micellar structures have been advocated as simple models for viruses and therefore, may have the ability of being effective drug carriers (Kataoka *et al.* 1992). These systems might be useful as pharmaceutical adjuvants of biologically active substances with a hydrophobic nature. Drugs may be physically trapped or incorporated within polymeric micelles. Indeed, a host of hydrophobic solutes have been solubilized in the interior of polymeric micelles. Alternatively a hydrophobic drug may be covalently bonded to the polyamino acid segment of an AB block copolymer within a micelle yielding a stable, solubilized system. Drugs solubilized in this manner may be released *in vivo* or *in vitro* systems as a result of dilution or/and destruction of micelles indirectly due to enzymatic degradation of polyamino acid segments within the core. Recently much interest has been focused on the development of polymeric micelles formed in aqueous media as drug delivery system (Kataoka *et al.* 1992 and 1993 Kwon *et al.* 1993a and b). AB block copolymers composed of PEG and polyamino acid have been designed based on the rationale that PEG expresses low interaction with proteins and cells and causes low toxicity, and the hydrophobic polyamino acid block would provide a biodegradable component as well as the required amphiphilicity for micellization. In principle, a systematic alteration in the structure of PEG-polyamino acid block copolymers through polymerization of different amino acids or through side chain modifications is attainable. These have apparent thermodynamic stability, and the ability to deliver drugs selectively *in vivo* (Kataoka *et al.* 1992, and 1993).

3.1.2 Liposomes

Liposomes are self-assembling colloidal particles in which a lipid bilayer encapsulates a fraction of the surrounding aqueous medium. Liposomes can entrap both hydrophilic and hydrophobic compounds, (Lasic, and Papahadjopoulos 1995) or interact with them in its bilayer structure. In general, hydrophobic compounds tend to remain in the phospholipid bilayer while hydrophilic molecules reside in the aqueous compartment of the liposome vesicles. Characteristics, such as gel-to-liquid crystalline transitions, permeability, partition coefficients, electrical properties, and elastic properties are important parameters in quality and quantity of drug delivery by liposome systems (Cullis, *et al.* 1987, Grit 1987, Dong and Rogers 1991, Fielding 1991). These properties can be adjusted by using mixtures of phospholipids (neutral and charged) or charged adducts and cholesterol in their composition (Gruner, 1987). Liposomes may be prepared from phospholipids using a number of techniques such as hydration, reverse-phase evaporation, detergent removal, and solvent injection methods. In general, the method used in the preparation of liposomes determines their size, lamellarity, and loading capacities.

3.2 Material and Methods

3.2.1 Materials

Cholesterol(CHOL, 99%), from Sigma Chemical Co., MO, USA, and L-α-dipalmitoyl- phosphatidylcholine (DPPC) from Nippon Oil Fats Co. Ltd., Japan were used as received. Poly(ethyleneglycol)-poly(beta-benzyl-L-aspartate) (Molecular weight 18000, PEG-PBLA) was received as a gift from Dr. G. Kwon (Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada). Taxol was purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA, and used as supplied. Water was deionized, Milli-Q (Millipore) purified. All other materials were reagent grade.

3.2.2 Preparation of PEG-PBLA Nanoparticles

Taxol (0.5-5 mg) and PEG-PBLA (0.5-20 mg) were dissolved in dimethylformamide (DMF, 2.0 ml), stirred for 3 hr, and dialyzed (molecular weight cut-off 10-12,000 g/mol) against 2×3L of distilled water at 37° C over 24 hr. The product was collected in a vial and its volume was adjusted to 10 ml with distilled water and then sonicated (micro tip probe, 40% output, pulsed, 10 min, bath temperature at 20° C) using sonicator W-375 (Heat Systems-Ultrasonics, Inc.), then left again over night to equilibrate at room temperature. The resulting samples were prepared for analysis. To study the effect of temperature on the process and overall solubilization effect, mixtures of taxol and PEG-PBLA in DMF were dialyzed against 2×3L of distilled water at 4, 25, 37, 50° C over 24 hr. The product was collected in a vial and its volume was adjusted to 10 ml with distilled water and then sonicated and then left again overnight to equilibrate at room temperature. To determine the effect of sonication on the solubilization of taxol, and the particle size of PEG-PBLA nanoparticle formulations, dialyzed samples were transferred to vials and after adjusting their volume to 10 ml they were sonicated for 0, 5, 10, and 20 min, and then particle size was measured (laser light scattering technique) then prepared for HPLC analysis.

3.2.3 Stability of PEG-PBLA Nanoparticles

Taxol:PEG-PBLA nanoparticles (1:5) containing 25 μg/ml taxol were transferred to screw capped vials. Then subjected to storage at different temperature (20, 37, 50, and 60° C). Screw-capped vials containing the solutions were placed at the desired temperature and once equilibration had been obtained, aliquots samples were drawn at appropriate time intervals and analyzed by HPLC.

3.2.4 Preparation of Liposomes

Lipid solutions (15 mM) containing L-α-dipalmitoylphosphatidylcholine:cholesterol (DPPC:CHOL, 2:1 molar ratio) were prepared. Volumes of 0, 0.5, 1, 2.5, 5, 10, and 20 ml of lipid solution were mixed with 1 ml of solution of taxol (1mg/ml) in *t*-butylmethyl ether then dried as a thin film in round-bottom flasks under reduced pressure using a rotary evaporator. These flasks were flushed with nitrogen gas, then kept overnight in a vacuum incubator at 37° C. Subsequently, the lipid film was hydrated with 10 ml of water. Dispersion and swelling of the lipid were performed at 10° C above the phase transition temperature of the phospholipids. The milky white suspension was vortex-mixed for 10 min, then allowed to stand for additional 2 hrs at room temperature, in order to complete the swelling process and to form multilamellar vesicles (MLVs). All samples were sonicated (micro tip, 40% output, pulsed, 10 min, bath temperature at 20° C) using sonicator W-375 (Heat Systems-Ultrasonics, Inc.), then left again overnight to equilibrate at room temperature. The resulting samples were centrifuged, filtered (Nalgene, 0.45 μm, 25-mm disposable syringe filter) and then prepared for analysis. To determine the effect of sonication on solubilization enhancement and particle size of liposome formulations, the hydrated film was sonicated for 0, 5, 10, and 20 min and particle size was measured (laser light scattering technique) then prepared for HPLC analysis.

3.2.5 Drug Assay

A Waters HPLC (Waters Lc module 1) consisting a Model 600E pump, a 715 auto-injector, and a 486 multi-wavelength UV detector (Waters, Mississauga, Canada) was operated at ambient temperature. A partisil ODS column (phenomenex ® 100 mm, 4.6 mm ID, 5 µm particle size), protected by a partisil ODS guard column (phenomenex ® 30 mm, 4.6 mm ID, 5 µm particle size) was used. The mobile phase consisted of 40% acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.0) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm. The resulting liposomes or PEG-PBLA nanoparticles were centrifuged, the supernatant was filtered (Nalgene, 0.45 µm, 25-mm disposable syringe filter) then 50 µl of each sample was directly injected in to the HPLC system.

3.2.6 Particle Size Determination

Liposome or nanoparticle solutions (100 µl) were diluted to 5 ml with water, then subjected to laser light scattering (Brookhaven Instruments BI-90 Particle sizer). The dust factor was maintained at 0.01 for all measurements.

3.2.7 Statistical Evaluation

Statistical evaluations were performed on all data using ANOVA and One-way ANOVA unless otherwise stated. Where a significant F-value was obtained, the Student-Newman-Keuls method was used for pairwise multiple comparisons, the level of significance chosen for all statistical analyses being $\alpha = 0.05$. The results were expressed as mean ± standard error of the mean (SEM).

3.3 Results and Discussion

3.3.1 PEG-PBLA Copolymer Systems

PEG-PBLA copolymers composed of PEG and polyaspartate have been designed based on the rationale that PEG and polyamino acid would provide biodegradable components as well as the required amphiphilicity for aggregation (Kataoka *et al.* 1992, Kwon *et al.* 1993a, Hagan *et al.* 1995). Figure 3-2 illustrates the extent of taxol solubilization in aqueous solutions as a function of PEG-PBLA concentration. The solubility of taxol increased in non-linear fashion as a function of PEG-PBLA. The solubility of taxol in PEG-PBLA system, was enhanced by increasing the amount of polymer from 0.5 to 5 mg reaching up to 56 μg/ml. Then falling to 34 μg/ml at 7 mg and increasing again to 45 μg/ml at 20 mg PEG-PBLA. The concentration of solubilized taxol decreased due, perhaps, to production of multi laminar vesicles (aggregates) or nanoparticles (> 450 nm) which could not pass through the filter. In the entrapping of hydrophobic compound in the interior of polymeric nanoparticles, one of the important parameters could be the concentration of hydrophobic solute at the aggregation time. Figure 3-3 shows the effect of increasing the amount of taxol in the aggregation media on overall solubilization of taxol by PEG-PBLA system. Decreasing the amount of taxol in aggregation media from 1 to 0.5 mg decreases the solubility of taxol by 1.7 fold, while increasing that from 1 to 2 mg only enhanced the solubility of taxol by 2% which is not significant. Increasing concentration of taxol to 3 and 5 mg decreases the solubility of taxol possibly due to hydrophobic interaction between taxol and core of the nanoparticles which may decrease the stability and entrapping capacity of nanoparticle.

The results show that temperature has a significant effect on the overall solubilization of taxol by PEG-PBLA systems (Figure 3-4). Increasing temperature from 5 to 25° C increased the solubilization of taxol from 29 to 56 μg/ml, respectively. After that, increasing the temperature reduced the solubility of taxol in PEG-PBLA solutions. Figures 3-5 and 3-6 show the effect of sonication on the solubility of taxol and particle size of nanoparticles in PEG-PBLA systems, respectively. Increasing the sonication time increased the solubility of taxol up to 10 min then remained constant. The best solubilizing results were achieved with taxol to a PEG-PBLA weight ratio of 3:20 and 20 min sonication time at 25° C. Under that condition maximum solubility of taxol was 120 μg/ml and average particle size was 98 nm.

The results of the stability studies of taxol in PEG-PBLA system showed that incorporation of taxol in the core of the nanoparticles protected taxol from degradation (Figure 3-7). The $t_{90\%}$ of taxol in water at 25° C is about 6 days while in PEG-PBLA system 93% of taxol is remained unchanged after 40 days. It can be concluded that PEG-PBLA system can increase the stability of taxol in aqueous media.

These studies showed that although incorporation of taxol in PEG-PBLA system is an effective method for increasing solubility and stability of taxol, optimization of taxol solubility is not a simple task and many parameters need to be considered.

3.3.2 Liposome Formulations

The maximum concentration of taxol solubilized in a given DPPC:CHOL system was determined by preparing the liposome formulation in the presence of excess amount of taxol and analysing the solubilized taxol after equilibration and filtration of liposome formulation and direct assay of taxol. Two parameters that could affect the solubilization potential of liposome formulation were studied: the total lipid concentration (DPPC + CHOL) and the effect of sonication. The molar ratio of DPPC:CHOL was 2:1 and kept constant in all cases. The amount of taxol solubilized increased significantly, and in a linear fashion as the total amount of lipid increased between 0-100 mg, verifying the hydrophobic interaction between taxol and phospholipid molecules (Figure 3-8). In this study sonication played the most dominant role in solubilization of taxol in liposome formulation. Usually after preparation of taxol by the hydration method many large multilamellar vesicles are produced. Although taxol was incorporated in liposome vesicles, these large particles could not pass through the filter (0.45 μm). Bath sonication could not decrease the particle size of these large vesicles efficiently. Applying harsh sonication method using micro tip probe decreased the particle size of these large vesicles to less than 200 nm which were capable to pass through filters. Figures 3-9 and 3-10 show the effect of sonication time on particle size and solubilized taxol in liposome formulation. There was a good correlation (r = 0.985), between particle size of liposomes and the concentration of taxol solubilized as a function of sonication time, the smaller the particle size of liposomes, the higher solubilization in liposomes. Although applying sonication for 20 minutes decreased particle size from 3200 nm to 187 nm (almost 16 fold reduction), solubility increased two fold which was a maximum (in this particular experiment the amount of taxol which was added to the system (100 μg/ml) was less than the solubility level). It seems that liposomes are able to increase the solubility of taxol to much larger extent than in PEG-PBLA systems.

The single point stability study of taxol in the liposome formulation showed that although the incorporation of taxol in liposomes protected taxol from degradation (95% of taxol remained unchanged after 15 days), however, because of aggregation, particle size growth, and sedimentation, the overall physical stability of the system is not as good as PEG-PBLA system. Our results revealed that liposome system is an effective approach for presenting taxol in aqueous media and delivery of taxol in short time.

3.4 Conclusions

Because of core/shell like and bilayer structures, PEG-PBLA nanoparticle and liposome systems can significantly increase solubility and stability of taxol in an aqueous formulation. In addition, biocompatibility of these systems offers a safe and convenient means for delivery of this remarkable anticancer drug by many routes of administration.

3.5 References

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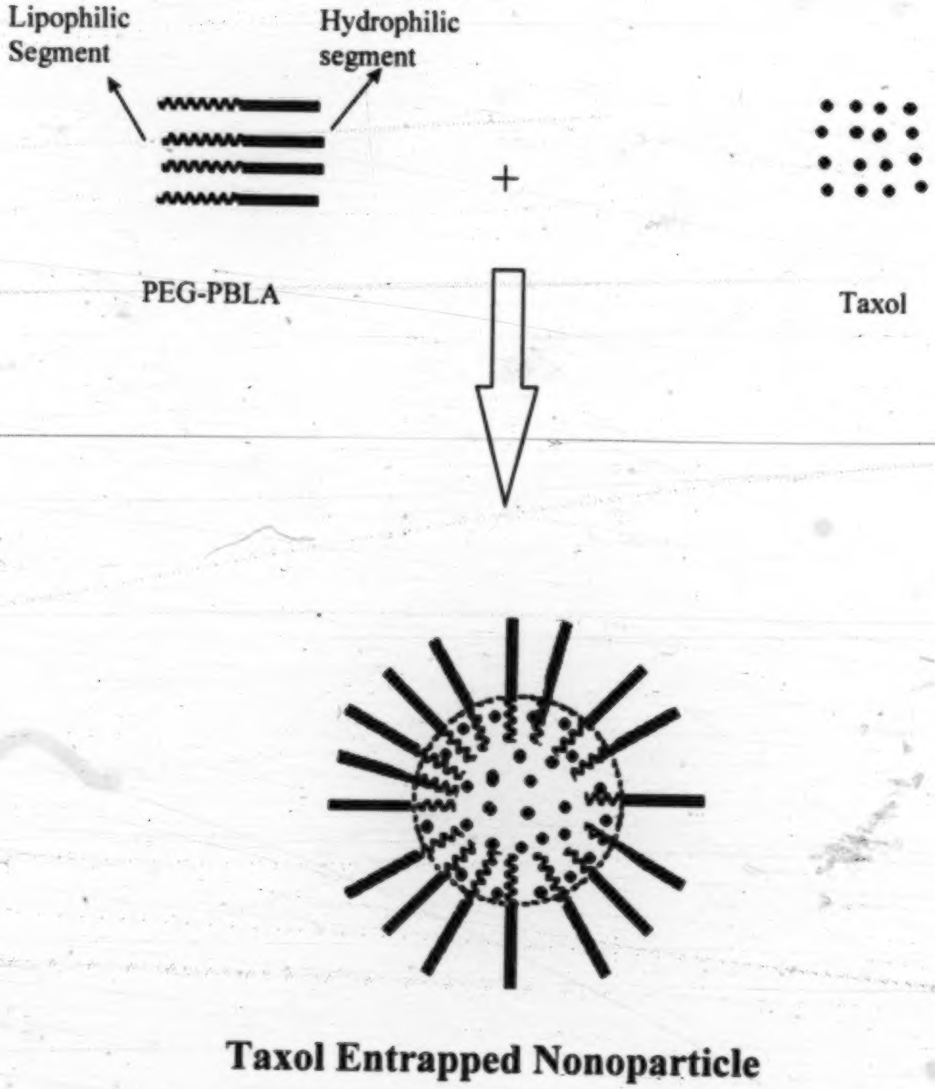

Lipophilic Segment
Hydrophilic segment
+
PEG-PBLA
Taxol
Taxol Entrapped Nonoparticle

Figure 3-1 Physical incorporation of taxol within PEG-PBLA nanoparticles

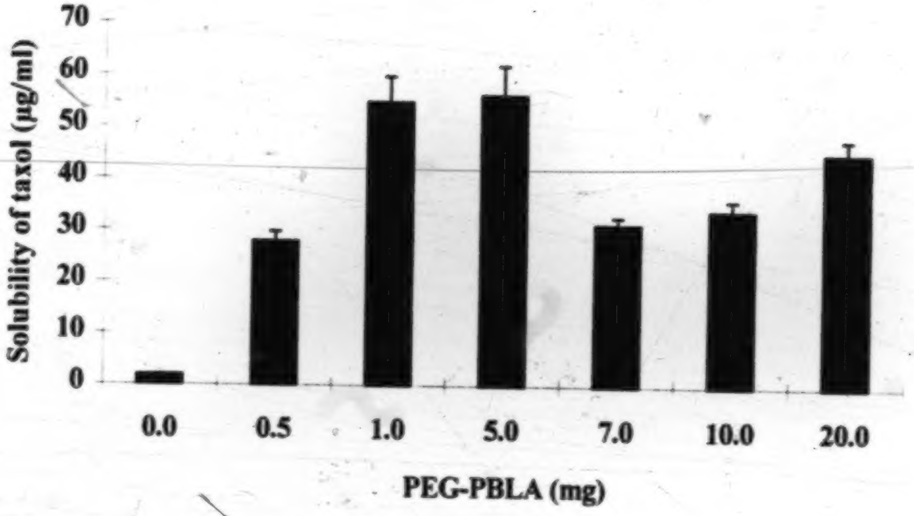


Figure 3-2 Solubility of taxol as a function of PEG-PBLA concentration (mg/10ml) at 25° C. The initial amount of taxol was 1 mg. Data are presented as the mean of three experiments ±SEM.

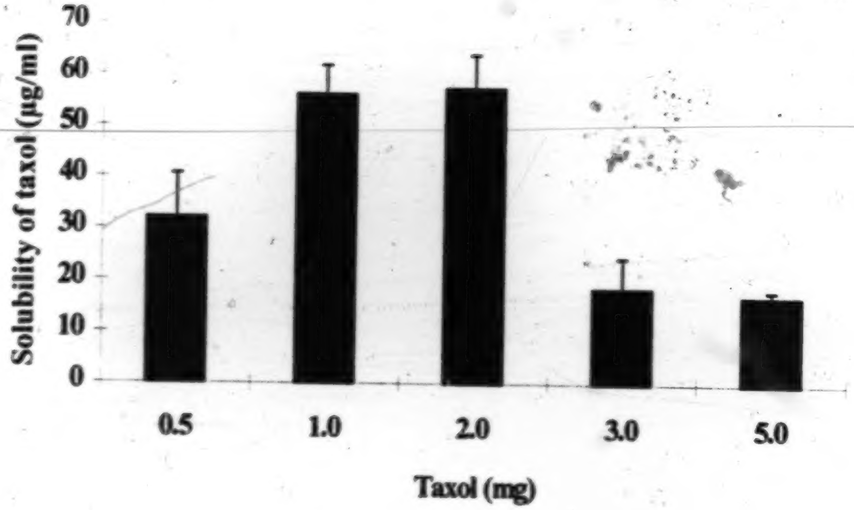


Figure 3-3 Effect of increasing the amount of taxol (mg/10 ml) in the aggregation media on the solubility of taxol. The initial amount of PEG-PBLA was 5 mg. Data are presented as the mean of three experiments ±SEM.

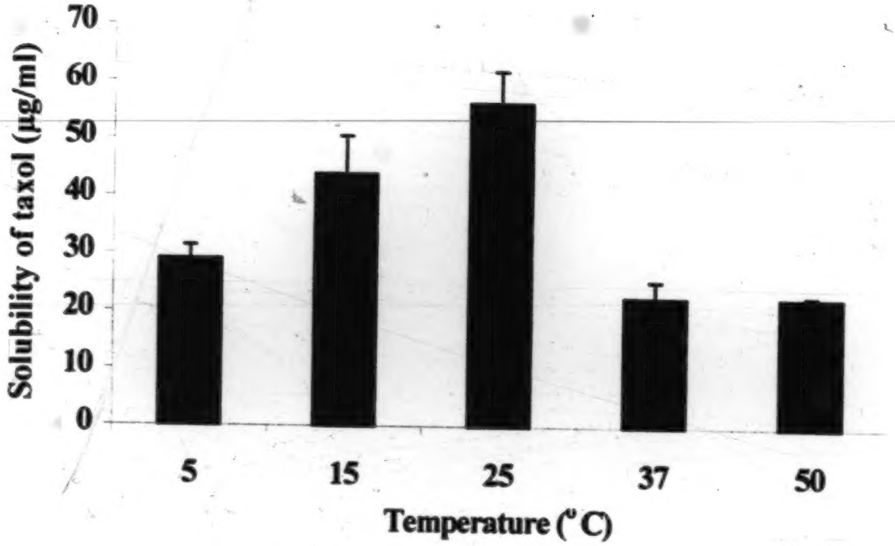


Figure 3-4 Comparing the effect of temperature (° C) on the solubility of taxol in a polymer media with taxol:polymer weight ratio of 1:5. Data are presented as the mean of three experiments ±SEM.

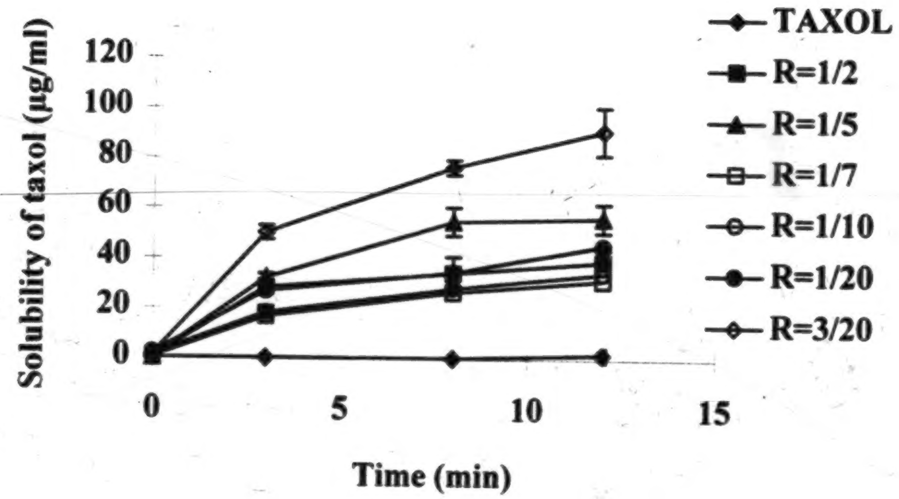


Figure 3-5 Effect of sonication time on the solubility of taxol in aggregation media containing different ratio of taxol:PEG-PBLA. Data are presented as the mean of three experiments ±SEM.

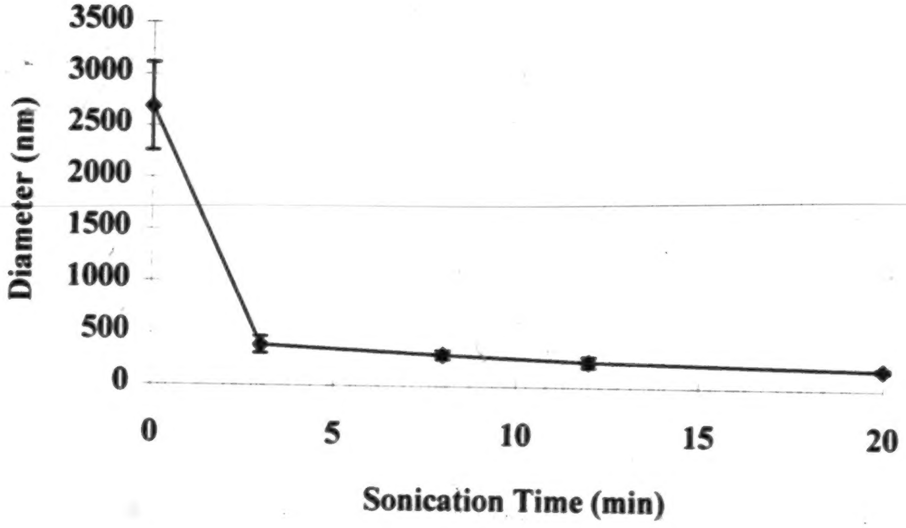


Figure 3-6 Effect of sonication time on particle size of nanoparticles containing taxol:PEG-PBLA (1:5 weight ratio). Data are presented as the mean ±SEM (n=3).

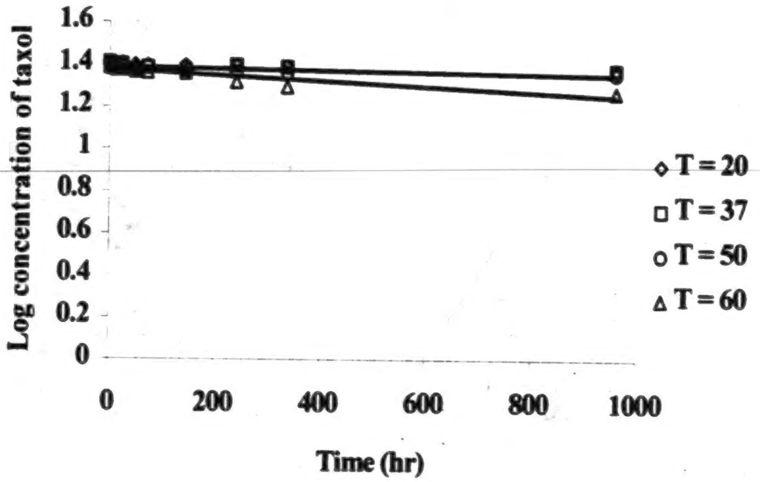


Figure 3-7 Stability of taxol at different temperature (° C) in PEG-PBLA nanoparticles containing 25 µg/ml taxol and taxol:PEG-PBLA ratio of 1:5. Data are presented as the mean ±SEM (n=3). Solid lines are the regression line through the data points.

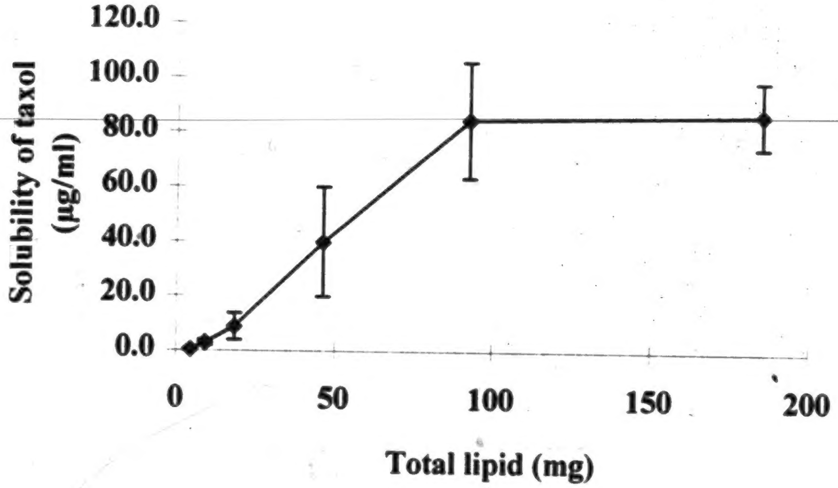


Figure 3-8 Solubility of taxol in liposome formulations containing different concentration (mg/10ml) of lipid (DPPC:CHOL, 2:1 molar ratio). Data are presented as the mean ±SEM (n=3). Solid lines are the regression line through the data points.

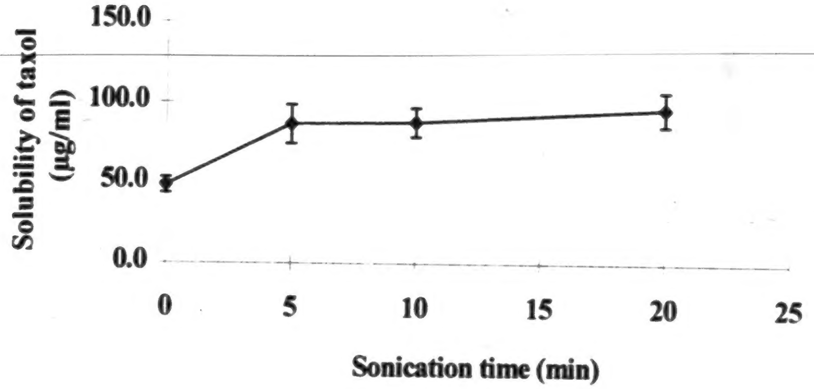


Figure 3-9 Effect of sonication time on the solubility of taxol in liposome formulation (DPPC:CHOL, 2:1 molar ratio and total amount of lipid 20 mg). Data are presented as the mean ±SEM (n=3).

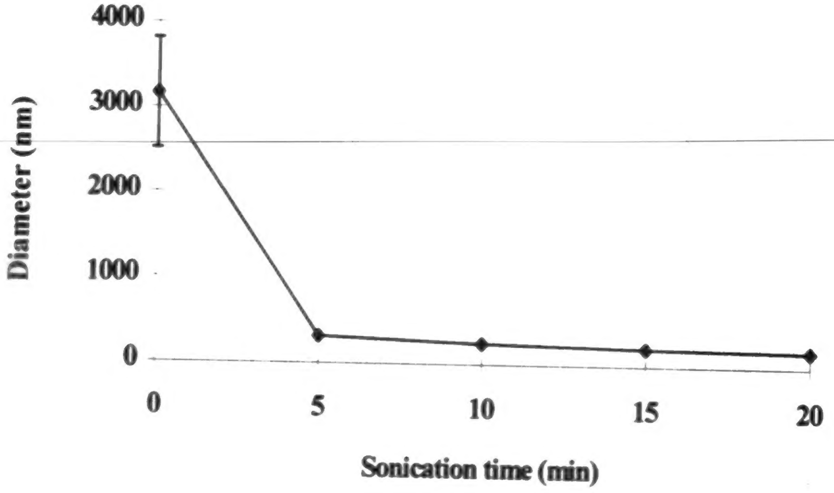


Figure 3-10 **Effect of sonication time on particle size of liposomes (DPPC:CHOL, 2:1 molar ratio and total amount of lipid 20 mg). Data are presented as the mean ±SEM (n=3).**

Chapter 4

4. Influence of Temperature, pH, Buffers, and Solubilizing Agents on the Stability of Taxol

4.1 Introduction

Clinical usage of taxol has been limited by its low solubility, stability, and toxic side effects (Koeller and Dorr, 1994). Taxol is susceptible to degradation processes such as mild basic and acidic hydrolysis, oxetan ring cleavage, and epimerization which results in the formation of baccatin III as the major product and several biologically active and inactive minor compounds (Ringel and Horwitz, 1987; Brown *et al.*, 1991). In hope of increasing taxol solubility and therapeutic efficacy, various taxol formulations have been developed, especially in solutions of hydroxypropyl-β-cyclodextrin (HPßCD), nicotinamide, polyethylene glycol 400 (PEG), bile salts (50:50 sodium cholate:deoxycholate), and cremophor EL (CR, cremophor:ethanol 50:50). Despite the clinical use of taxol in treatment of cancer, very little information on the stability of taxol is available. An increasing interest in developing a new formulation of taxol has heightened the need to understand the basic physicochemical properties of taxol in order to facilitate the formulation of liquid product. As part of continuing effort in our laboratory to further understanding of the physicochemical properties of taxol, the solution stability of taxol was followed as a function of temperature, pH, ionic strength, buffer components and solubilizing agents.

4.2 Materials and Methods

4.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Co, (USA), and used as supplied. Hydroxypropyl-ß-cyclodextrin (molecular weight=1380) was obtained from Aldrich Chemical company (Milwaukee, WI, USA), and was used without further purification. Nicotinamide, polyethylene glycol 400, and bile salts (50:50 sodium cholate:deoxycholate), were supplied from Sigma Chemical Company (St. Louis, MO, USA). All other materials were reagent grade. Water was deionized and passed through a Milli-Q apparatus (Millipore).

4.2.2 Stability Determination

The stability studies were carried out by adding a stock solution of taxol in cremophor EL (CR, cremophor:ethanol 50:50) to: 1) water; 2) aqueous solutions of citrate buffers (pH values of 1.2 and 2.5) and phosphate buffers (pH values 4.5, 6.5, 7.4, and 8) ionic strength of all was adjusted with sodium chloride to 0.5; 3) aqueous solutions of HPβCD (concentration ranged from 0.5 to 20% w/v); 4) nicotinamide (20% w/v); 5) PEG (20% w/v); and 6) bile salts (20% w/v). Before addition of taxol all solutions were equilibrated at the desired temperature (20, 37, 50, and 60° C) in a water bath, and after addition of taxol they were mixed thoroughly. The effect of buffer concentration (0.025, 0.05, and 0.1 M) on the degradation of taxol was studied in phosphate and citrate buffers. The influence of different ionic strength (0.1, 0.25, and 0.5) on the degradation of taxol was studied in 0.025 M phosphate buffer (pH 5.5) at 37° C. The initial taxol concentration was 10 μg/ml. The CR concentration in the final reaction mixture was less than 0.5%. Screw-capped vials containing the test solutions were placed at the desired temperature. Aliquots samples were withdrawn at appropriate time intervals and processed as described in the assay under analysis of samples.

4.2.3 Method of Analysis of Samples

Analyses were carried using an HPLC system consisting of (Waters Lc module 1. 600E pump, 715 auto-injector, and 486 UV detector), with a partisil ODS column (phenomenex ® 100 mm,4.6 mm ID, 5 μm particle size) protected by a partisil ODS guard column (phenomenex ® 30 mm, 4.6 mm ID, 5 μm particle size), using a mobile phase of 43% acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.5) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm. Samples of 1 ml plus 0.1 ml of internal standard (fenoprofen 3.5 μg/ml in acetonitrile 47% in water) were extracted with 3.5 ml of *t*-butyl methyl ether and vortex-mixed for 1 min. The mixture was then centrifuged for 10 min at 1100 g after which 3 ml of the organic layer were removed and evaporated to dryness under vacuum at low temperature. The residue was reconstituted in 0.2 ml of 47% aqueous acetonitrile and 50 µl was injected in the HPLC system. Standard solutions of taxol were made when appropriate, extracted and injected before, during, and after a series of samples were injected. Data are presented as the mean of three experiments.

4.3 Results and Discussion

4.3.1 Determination of First-Order Rate Constants

At constant temperature and ionic strength, the loss of taxol in the presence of aqueous buffers and solubilizing agents was best described by first-order kinetics. apparent first-order rate constants (k_{obs}) were calculated from linear first-order plots based on:

$$\log [C] = \log [C_o] - (k \times t / 2.303)$$

where the concentration is [C], the initial concentration is $[C_o]$, and time is t. Rate constants (Table 4-1) were obtained from linear regressions.

4.3.2 Effect of Buffer Concentration

Figure 4-1 displays the effect of increasing buffer concentration (0.025-0.1 M) on the degradation rate constant of taxol at 37° C at pH values of 1.2, 2.5, (citrate buffers) 4.5, 6.5, 7.4, and 8.0 (phosphate buffers). Since the slope of this line was almost zero, the buffer concentration effect was interpreted to be negligible on the degradation kinetics of taxol in that range. The higher value of the degradation rate constant in the 0.1 M solution of phosphate buffer at pH 8 may be due to day-to-day variation in the assay for this particular sample. Therefore, the k_{obs} in citrate and phosphate buffers at 0.025 M (which is ≈ to zero concentration of buffer) were assumed to be the degradation rate constant at that specific pH.

4.3.3 Effect of Ionic Strength

At constant temperature (37° C) and buffer concentration (0.05 M), the effect of ionic strength on the degradation of taxol at pH 4.5 was studied and the rate constant in infinitely dilute solution (k_o) in which $\mu = 0$, was calculated based on:

$$\log k_{obs} = \log k_o + C\mu$$

in which C is a constant obtained from experimental data (Zhou, and Notari 1994). Figure 4-2 shows the effect of ionic strength on the degradation of taxol in 0.05 (M) phosphate buffer (pH 4.5) at 37° C. Since the slope, C, of a plot of log k_{obs} versus ionic strength was -0.858, the ionic strength was interpreted to be an important factor on the degradation kinetic of taxol. These data suggest that the degradation rate of taxol decreases with increasing ionic strength of the buffers. The y-intercept of that plot represents the rate constant in infinitely dilute solution (k_o) which was equal to 0.054 hr^{-1}.

4.3.4 pH-Rate Profile

The influence of the pH on the degradation of taxol is demonstrated in figure 4-3. Figure 4-1 indicates that buffer concentration has a negligible effect on the degradation kinetics of taxol. Therefore, log of the k_{obs} at 0.025 M buffer concentration at 37° C was plotted as a function of pH. The profile shows a specific acid catalytic region at pH below 2.5, a plateau of pH-independent degradation between pH 3 and 6, and a sharp increase in degradation rate at pH above 7. In the plateau region (pH 4.5), the activation energy of (E_a) was determined to be 2.4 Kcal/mol (Table 4-1). Although the exact intramolecular mechanism of degradation of taxol is still unclear, the small negative entropy of activation (-0.003 Kcal/mol/deg) may be a characteristic of unimolecular reaction (Loftsson *et al.* 1992, bimolecular reactions usually result in a much larger negative entropy). Therefore, it was concluded that the instability of taxol at pH 4.5 is due to a unimolecular reaction occurred through possible epimerization of taxol to 7-epitaxol (Figure 1-4, Ringel and Horwitz, 1987).

4.3.5 Effect of Buffer Component

Figure 4-4 illustrates the degradation of taxol in phosphate, acetate, and citrate buffers with the same pH, ionic strength, and concentration at 37° C. It seems that decomposition of taxol in citrate is 4 and 7 times faster than acetate and phosphate buffers respectively.

4.3.6 Effect of Temperature

Figure 4-5 shows the Arrhenius plot for the degradation of taxol in water. The logarithm of rate constant at the lowest buffer concentration studied (0.025 M) was plotted against the reciprocal of the temperature in Kelvin degrees to determine the temperature dependency of buffer catalytic constant of taxol (Arrhenius expression, Connors 1990). The data from the lowest buffer concentrations were used in all of the estimation, in order to predict a shelf life in the presence of a weak buffer. A linear regression was performed on the data using the 0.025 M buffer concentration and the energies of activation were calculated from the slopes. The value of the observed degradation rate constant was extrapolated to 25° C and the shelf life of 74 hrs or more was predicted by these estimates, at a pH value of 6.5. All calculations were made based upon the assumption that the energy of activation does not change with temperature. The energy of activation in pH range of 4.5 to 6.5 was calculated to be between 3.28 to 7.79 Kcal/mol, and it demanded a detailed investigation of various approaches to stabilize the cremophor formulation of taxol.

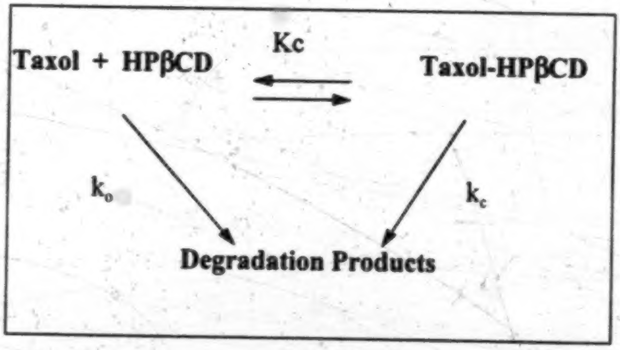
4.3.7 Solubilizing and Stabilizing Agents

4.3.7.1 Cyclodextrins

Cyclodextrins can substantially increase the stability of compounds through their ability to form inclusion complexes with many drugs. Improvement of stability of prostaglandin E1(Yamamoto *et al.* 1992), psoralen (Vincieri, *et al.* 1995), nitrazepamm (Saleh, *et al.* 1993), mitomycin (Bekers *et al.* 1991 and 1993), tauromustine (Loftsson

1992), 2', 3'-dideoxyinosine (Bekers *et al.* 1993), by cyclodextrin complexation have been reported.

The observed rates of degradation of these compounds were significantly reduced by complexation with 2-HPβCD and γCD, depending on the structure of the compound. These studies suggest that cyclodextrin complexation may be a useful approach to stabilize taxol without loss of its pharmacological activity. Taxol was found to form an A_L type inclusion complex with HPβCD in solution. The effects of temperature and concentration of HPβCD on the stability of taxol in HPβCD solutions were studied. The apparent-first-order rate constants (k_{obs}) were determined from the disappearance of the drug by linear regression of the logarithm of the concentration of the drug remaining versus time. Degradation of taxol in HPβCD followed first-order kinetics and introduction of up to 20% (w/v) HPβCD to the reaction medium did not affect this kinetic behavior, as linear relationship ($P<0.05$) was in all cases obtained between the logarithms of the concentration of the drug remaining and time (Figure 4-6). Degradation of taxol, both within the different media, or water, increased progressively with elevation of temperature in all solutions of taxol in HPβCD (Figure 4-7). The kinetic parameters for the degradation of taxol are displayed in Table 4-2. Comparison of the rate constants shows that all HPβCD solutions tested (concentration of HPβCD: 0-20% w/v), have significant effect on stability of taxol. Increasing the HPβCD concentration in the reaction medium decreased the rate of degradation of taxol (18.5 times slower in 20% w/v HPβCD), and a non-linear relationship was obtained between the apparent-first-order rate constants and HPβCD concentration (Figure 4-8). This type of dependence of the rate constant on the HPβCD concentration is characteristic of saturation kinetics where the drug degrades at slower rate within the cyclodextrin complex than outside of it (Scheme 1, Loftsson *et al.*, 1991). The enthalpy for the complex formation is negative, resulting in a decrease in free energy due to the complexion process. On the other hand, the activation parameters for the degradation result in an increase in the free energy. Thus, when the temperature of the HPβCD containing reaction medium is lowered, both decrease in the degradation rate constant and increase in the stability constant for the inclusion complex will result in stabilization of taxol.



Scheme 1 **Relationship between the total HPβCD concentration and the observed rate constant for degradation of taxol.**

k_o represents the apparent-first-order rate constant for degradation of free taxol (hr^{-1}),

k_c represents the apparent-first-order rate constant for degradation of taxol in the complex (hr^{-1})

K_c represents the stability constant for the complex, assuming 1:1 complex formation (M^{-1}).

Figure 4-8 shows the relationship between log degradation rate constant of taxol in HPßCD solution as a function of 1/T. As can be seen there is a deviation from the Arrhenius plot at higher temperatures which may be due to changes in the association of HPßCD molecules in solution and consequential interaction with taxol.

4.3.7.2 Nicotinamide

Nicotinamide, 3-pyridine carboxamide, is a nontoxic B vitamin (vitamin B_3) that has been shown to enhance the aqueous solubility of many drugs including taxol through complexation. The exact mechanism by which this compound forms complexes is not entirely clear, but it has been suggested that it could be the result of a *Pi*-donor *Pi*-acceptor interaction (Hussain *et al.* 1992, Rasool *et al.* 1990). Taxol has a carbonyl group and a Pi-conjugated system in its molecular structure and it interacts with nicotinamide in a nonlinear fashion as a function of pH and nicotinamide concentration. Our previous results have shown that the solubility of taxol was enhanced with increasing nicotinamide concentration (0.5 to 20% w/v) up to 95-fold, compared to water (0.32 mg/L), and at pH 4.5 a maximum solubility of 59 mg/L of taxol was observed in 20% w/v nicotinamide solution. The effect of temperature on the drug stability of taxol in nicotinamide solution was studied. The pseudo-first-order rate constants (k_{obs}) determined from the disappearance of the drug by linear regression of the logarithm of the concentration of the drug remaining versus time. Degradation of taxol both within the nicotinamide or water increased progressively with elevation of temperature in all solutions of taxol in nicotinamide (Figure 4-9). Comparison of the rate constants (Table 4-2) shows that nicotinamide has significant effect on the stability of taxol. Taxol degraded 1.7 times faster in 20% nicotinamide than water. Nicotinamide in aqueous solution is almost neutral in reaction, although complexation of nicotinamide and taxol can increase the solubility of taxol, this complexation apparently does not protect taxol from degradation. Figure 4-9 shows the relationship between log degradation rate constant of taxol in nicotinamide solution as a function of 1/T. As can be seen there is a deviation from the Arrhenius plot at higher temperatures which may be due to changes in the association of nicotinamide molecules in solution and consequential interaction with taxol.

4.3.7.3 Bile Salts

The main products of cholesterol metabolism are bile salts which are, biologically, the most important detergent-like molecules. Bile salts are amphipathic molecules, possessing hydrophilic and hydrophobic regions which enable them to display characteristic behavior in water. The surface active and self-association properties of bile salts allow them to form micelles which solubilize poorly water-soluble drugs (Coello *et al.* 1996). Bile salts have been reported to increase the apparent solubility of many poorly water-soluble drugs such as diazepam, griseofulvin, NSAIDs, 1,8-dinitropyrene, triamcinolone, betamethasone, and dexamethasone (Bates *et al.* 1966, Rosoff *et al.* 1980, Miyazaki *et al.* 1981, Coello *et al.* 1996). Incorporation into micelles appeared to increase solubility of these compounds at concentrations above critical micelle concentration of bile salts (13-16 mM or higher). Our previous results have shown that solubility of taxol increased 45-fold in a nonlinear fashion with increasing bile salt concentration (0.5 to 20% w/v) in the medium. In this study the degradation kinetics of taxol in solutions of bile salts (50:50 sodium cholate:deoxycolate), were investigated. The apparent-first-order rate constants (k_{obs}) determined from the disappearance of the drug by linear regression of the logarithm of the concentration of the drug remaining versus time. Degradation of taxol both within the bile salts or water increased progressively with elevation of temperature in all solutions of taxol in bile salts (Figure 4-10). Comparison of the rate constants (Table 4-2) shows that bile salts have a significant effect on stability of taxol. Our results revealed that taxol degraded 12.6 times faster in 20% bile salts than water. The pH of bile salts solutions (concentration 0.5-20% w/v) in aqueous solution is alkaline (pH > 8). Although micellization of bile salts can increase solubility of taxol, this micellization can not protect taxol from alkaline degradation. Therefore, low stability of taxol in bile salts solutions could be due to a high pH of bile salt solutions and the presence of specific base catalytic degradation of taxol at pH above 7.

4.3.7.4 Polyethylene Glycol

Organic cosolvents are among the most powerful solubilizing agents for a large number of lipophilic drugs (Li and Yalkowsky 1994). Our previous results have shown that the solubility of taxol increased dramatically in a linear fashion with increasing polyethylene glycol 400 (PEG) concentration (0.5 to 100% w/v) in the medium. The influence of PEG on the stability of taxol was studied using a mixture of PEG:water, (20%). Also the effect of temperature on the drug stability of taxol in PEG solution was studied. The apparent-first-order rate constants (k_{obs}) determined from the disappearance of the drug by linear regression of the logarithm of the concentration of the drug remaining versus time. Degradation of taxol, both within the PEG as well as water increased progressively with elevation of temperature in all solutions of taxol (Figure 4-11). Comparison of the rate constants (Table 4-2) shows that PEG had a significant effect on the stability of taxol. Taxol degraded 1.7 times slower in 20% PEG than water. PEG in aqueous solution is almost neutral in reaction. The presence of PEG not only increased solubility of taxol, but it also offered some protection of taxol from degradation. Replacement of water by PEG up to 20% v/v may stabilize taxol against possible hydrolysis. However, the exact mechanism of protection is not clearly understood and further studies are required. Figure 4-11 shows the relationship between degradation rate constant of taxol in PEG solution as a function of 1/T. As can be seen there is a deviation from the Arrhenius plot at higher temperatures which may be due to changes in the association of PEG molecules in solution and consequential interaction with taxol.

4.4 Conclusions

The results show that all solubilizing agents had significant effect on the stability of taxol. However, PEG and HPβCD not only increased the solubility of taxol but they also stabilize taxol against degradation in aqueous solutions. The HPβCD stabilizing effects depended on the concentration of HPβCD and the pH of the median.

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Table 4-1 Kinetic parameters of degradation of taxol in water and in the presence of different buffers at 37° C

Parameters	pH = 1.2	pH = 2.5	pH = 4.5	pH = 6.5	pH = 7.4	pH = 8	WATER
k_{obs} (hr^{-1}) $\times 10^3$	16.9	1.90	1.10	2.70	2.60	15.4	0.14
$t_{1/2}$ (hr)	41.0	357	647	259	270	44.9	487
$t_{90\%}$ (hr)	6.20	54.3	98.4	39.3	41.0	6.80	74.0
E_a (Kcal/mol)	12.7	6.20	2.40	7.50	20.30	5.40	4.20
$\Delta G°$ (Kcal/mol)	2.49	3.79	4.20	3.61	3.73	2.67	4.01
$\Delta S°$ (cal/mol/deg)	43.6	16.2	-3.20	13.2	11.7	55.9	19.9

Table 4-2 Calculated kinetic parameters of degradation of taxol in water and in the presence of different solubilizing agents for 25° C

Parameter	PEG 400	HPβCD	Nicotinamide	Bile Salts	Water
$k_{(obs)}$ (hr^{-1})	2.88×10^{-4}	4.02×10^{-5}	1.33×10^{-3}	0.09	7.44×10^{-4}
$t_{1/2}$ (day)	100	719	21.7	0.31	38.8
$t_{90\%}$ (day)	15.3	109	3.29	0.05	5.96
E_a (Kcal/mol)	11.1	10.1	8.35	2.24	6.17
$\Delta G°$ (Kcal/mol)	4.83	5.99	3.90	2.77	4.27
$\Delta S°$ (cal/mol/deg)	-46.5	-53.8	-39.0	-11.3	-38.6

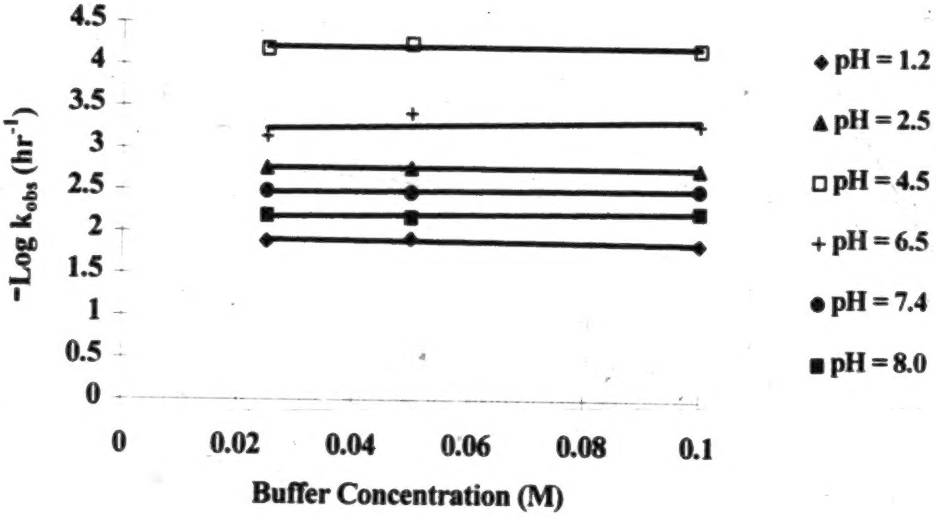

Figure 4-1 Effect of increasing buffer concentration on the degradation rate constant of taxol at 37° C and different pH (1.2-8.0), $\mu = 0.5$. Solid line is the regression line through the data points.

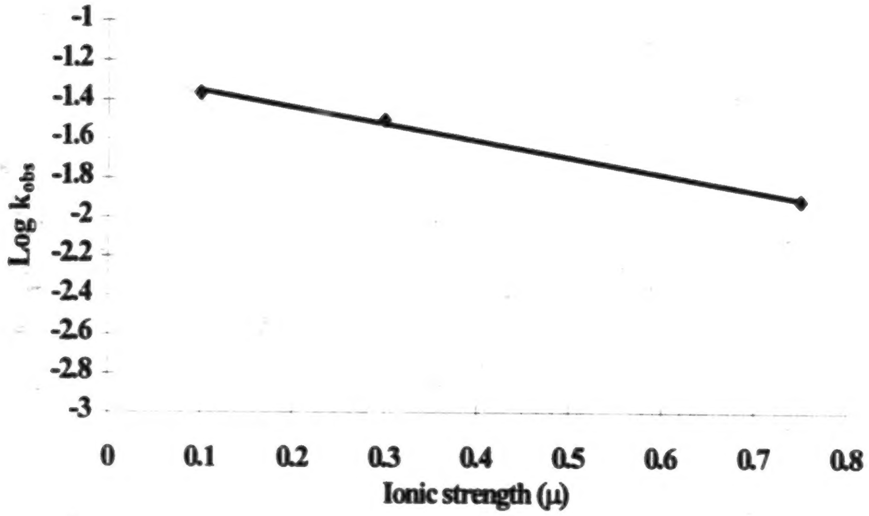

Figure 4-2 Effect of ionic strength on the degradation rate constant of taxol in 0.05 M phosphate buffer at 37° C and pH 4.5. Ionic strength was adjusted with NaCl. Solid line is the regression line through the data points.

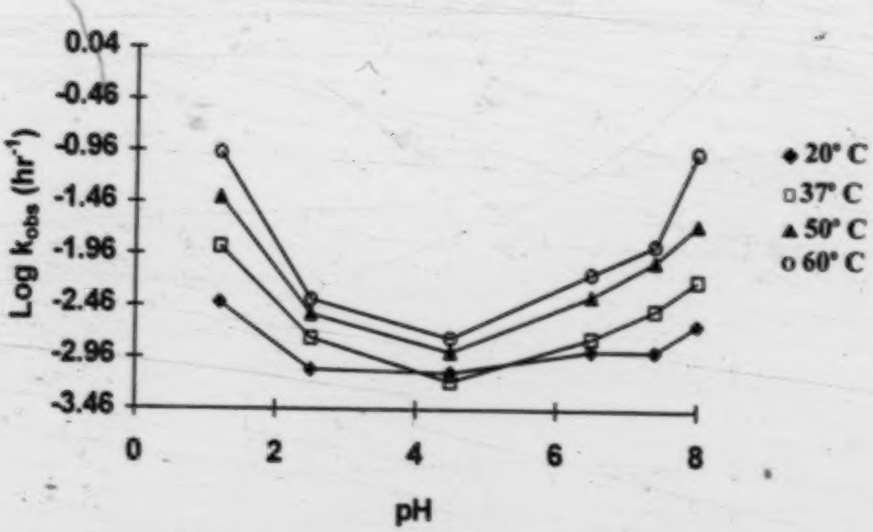

Figure 4-3 pH-rate profile for the degradation of taxol at different temperatures at an ionic strength of 0.5. Data are presented as the mean of three experiments.

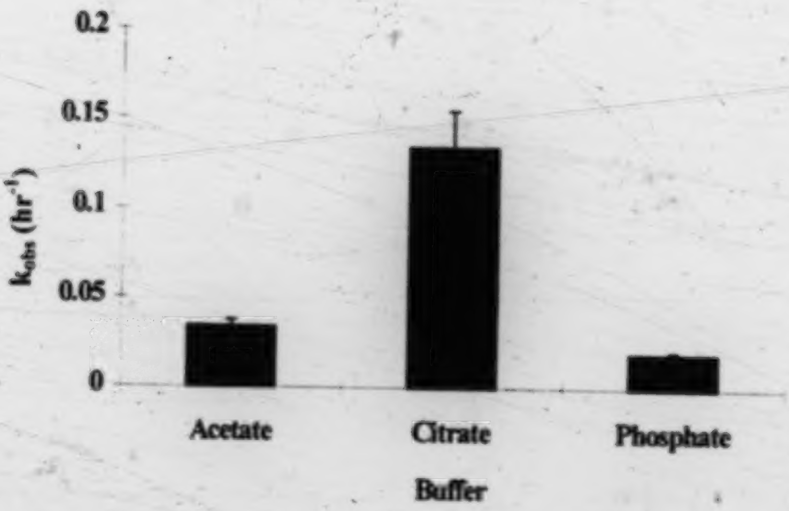

Figure 4-4 Effect of buffer component on the degradation rate constant of taxol in different buffer at 37° C (pH = 4.5, C = 0.05 M, and μ = 0.5). Data are presented as the mean ±SEM (n=3).

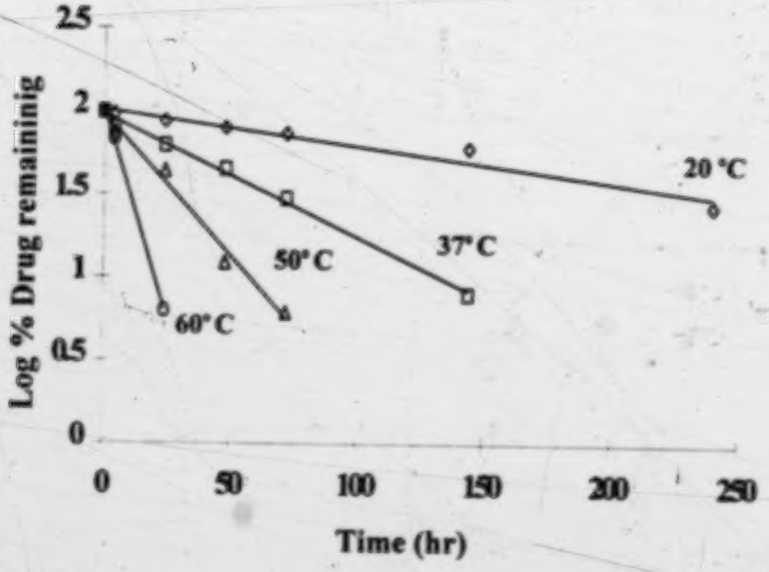

Figure 4-5 Accelerated decomposition rate of taxol at pH 1.2 at elevated temperature. Solid lines are regression lines through the data points.

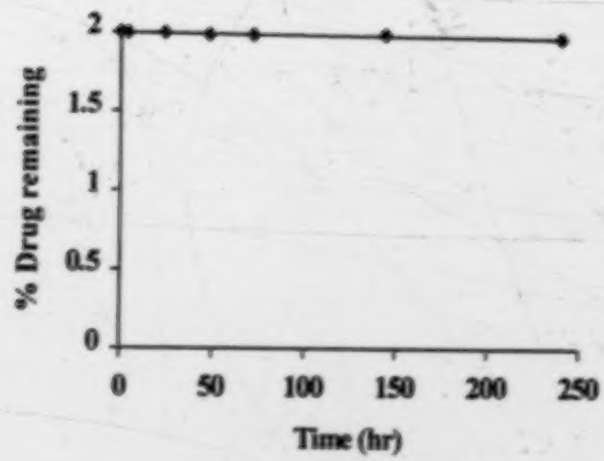

Figure 4-6 Decomposition of taxol in the presence of HPBCD solution (5% w/v) at 20° C. Solid line is the regression line through the data points.

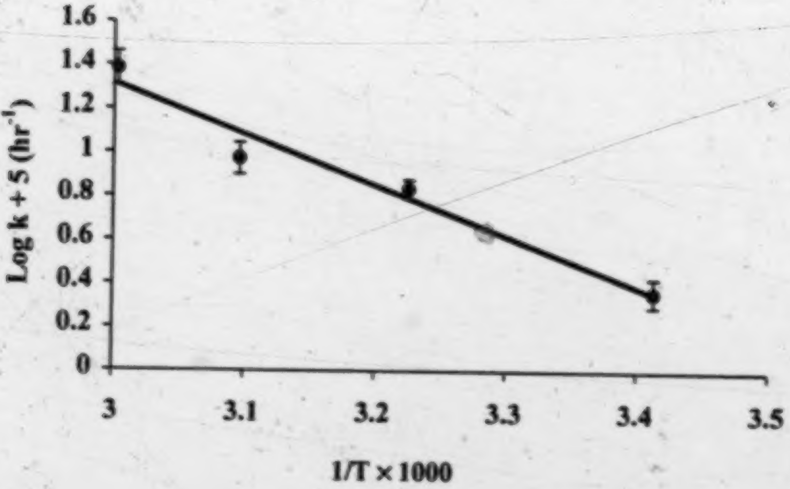

Figure 4-7 A plot of log k against 1/T of taxol in HPβCD solution (20% w/v). Solid line is the regression line through the data points. Log k + 5 is plotted on the vertical axis to eliminate the negative values along the axis.

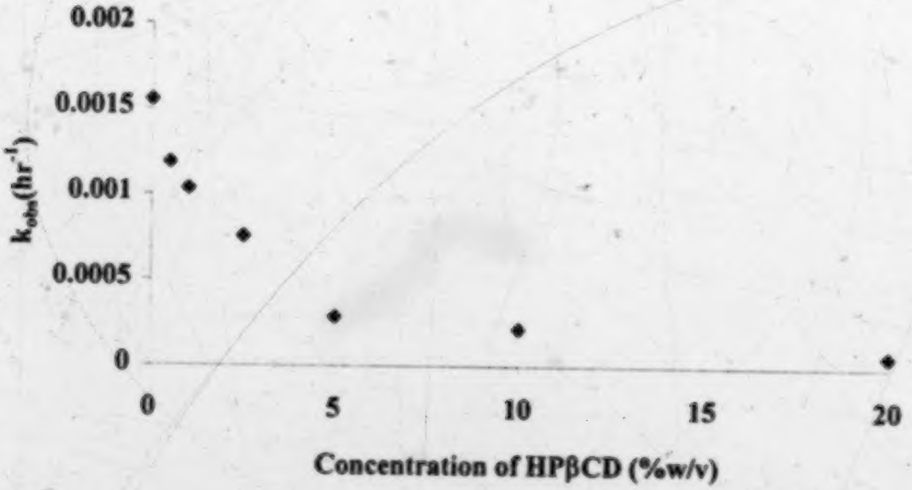

Figure 4-8 The relationship between k_{obs} and HPβCD concentration at 20° C.

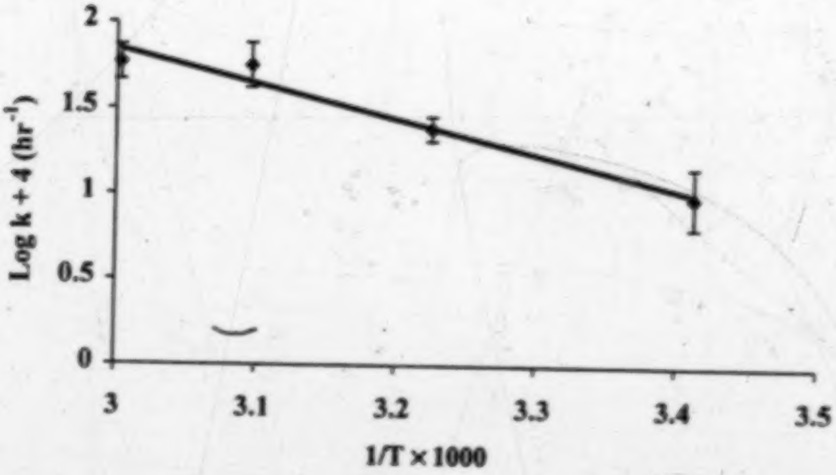

Figure 4-9 A plot of log k against 1/T of taxol in nicotinamide solution (20% w/v). Solid line is the regression line through the data points. Log k + 4 is plotted on the vertical axis to eliminate the negative values along the axis.

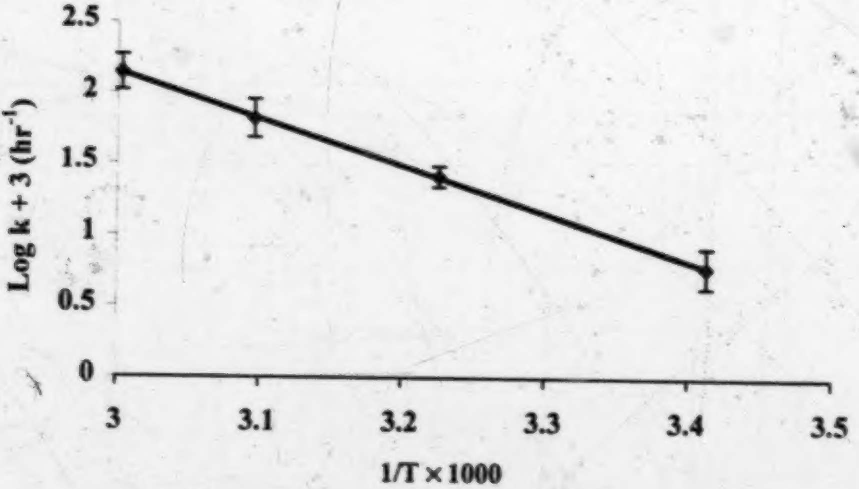

Figure 4-10 A plot of log k against 1/T of taxol in bile salts solution (20% w/v). Solid line is the regression line through the data points. Log k + 3 is plotted on the vertical axis to eliminate the negative values along the axis.

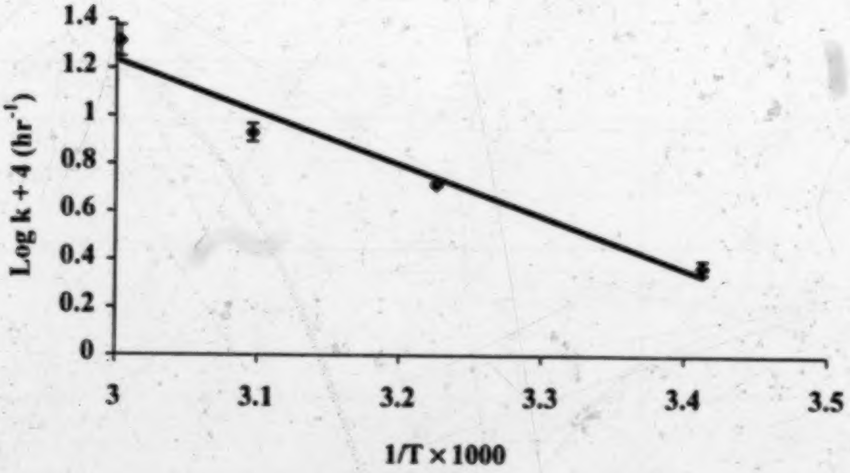

Figure 4-11 A plot of log k against 1/T for the thermal stability of taxol in PEG solution (20% w/v). Solid line is the regression line through the data points. Log k + 4 is plotted on the vertical axis to eliminate the negative values along the axis.

Chapter 5

5. Evaluation of the Dissolution Characteristics of Taxol

5.1 Introduction

The rate and extent of absorption of a drug administered orally is controlled by many factors, among which the dissolution rate is the most important parameter. Taxol, which is one of the more intriguing new anticancer drugs to enter the clinical arena in many years, has shown a very low and variable bioavailability (Koeller and Dorr, 1994). In order for a drug to be absorbed to any appreciable extent across the gastrointestinal barrier, it must be in a solution form. Thus for poorly water-soluble drugs, one of the rate-limiting steps in the absorption process is often the dissolution behavior in the gastrointestinal tract. Based on the Noyes-Whitney type of expression of initial dissolution rate of powder,

$$dc/dt = [D \times A \times (C_s - C_t)] / V \times h \qquad \text{Eq (1)}$$

The dissolution rate dc/dt is a function of the surface that is available for dissolution, A, the saturation solubility of the compound, C_s, the diffusion coefficient of the compound, D, the volume of the dissolution media, V, and the boundary layer thickness, h. C_t is the concentration at time t. It has been shown that formulation factors, mainly, type of excipient, can affect the solubility and, thus, the dissolution rate, bioavailability and the incidence of side effects of drugs in animals and humans. Therefore, in the present study, in order to elucidate the extent by which the dissolution rate of taxol may be improved, the dissolution behaviors of taxol in water, buffer solutions and solutions of solubilizing agents were evaluated using oscillating-bottle Method.

5.2 Materials and Methods

5.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA, and used as supplied. The following cyclodextrins were obtained from Aldrich Chemical Company, Milwaukee, WI, USA, and were used without further purification: α-cyclodextrin β-cyclodextrin, and hydroxypropyl-ß-cyclodextrin (MW = 1380).

Nicotinamide and bile salts were supplied by Sigma Chemical Company, St. Louis, MO, USA. Gamma-cyclodextrin was obtained from American Maize-Products company, Hammond, IN, USA. Water was deionized, Milli-Q (Millipore) purified. All other materials were reagent grade.

5.2.2 Dissolution Method

The dissolution studies were carried out by adding powdered taxol to water, buffered aqueous solutions (in pH values of 1.2, 2.5, 4.5, citrate buffers and 6.5, 7.4, and 8.0 in phosphate buffers) or aqueous solutions of γCD, HPβCD, nicotinamide, and bile salts, in 100-ml flasks previously equilibrated at desired temperature of 37° C in a water bath, and were shaken at 50 rmp. Aliquots (1 ml) of the solution were withdrawn at appropriate time intervals through a 0.45-μm filter and processed as directed in the assay of taxol. The resultant loss in volume was compensated by adding the dissolution medium maintained at the same temperature.

In order to compensate for the previously removed samples, a cumulative correction was made using the following equation:

$$C_n = C'_n + (V_s/V_d) \times C_{n-1} \qquad \text{Eq (2)}$$

where C_n is the expected concentration of the nth sample if previous samples had not been removed, C'n is the measured concentration, C_{n-1} is the expected concentration in the previous sample, V_s is the sample volume, and V_d is the dissolution medium volume. In addition, the dissolution of taxol in water was studied at different temperature (25, 37, 50, and 60° C). The initial taxol concentration was less than the saturation level of taxol in each media. Dissolution rates were calculated from the linear portion of the concentration-vs-time plots.

5.2.3 Method of Analysis of Samples

Samples were analyzed for taxol using a Waters HPLC system (Lc module 1) consisting of a model 600E pump, a model 715 auto-injector, and a model 486 UV detector, with a partisil ODS column (phenomenex ® 100 mm, 4.6 mm ID, 5 μm particle size) protected by a partisil ODS guard column (phenomenex ® 30 mm, 4.6 mm ID, 5 μm particle size), using a mobile phase of 43% acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.5) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm. Samples of 1 ml plus 0.1 ml of internal standard (fenoprofen 3.5 μg/ml in acetonitrile 47% in water were extracted with 3.5 ml of *t*-butyl methyl ether and vortex-mixed for 1 min. The mixture was then centrifuged for 10 min at 1100 g after which 3 ml of the organic layer were removed and evaporated to dryness under vacuum at low temperature. The residue was reconstituted in 0.2 ml of 47% aqueous acetonitrile and injected (50 μl) into the HPLC system. Standard solutions of taxol were made when appropriate, extracted and injected before, during, and after a series of samples were injected. Data are presented as the mean of three experiments.

5.3 Results and Discussion

5.3.1 Dissolution of taxol in Water and Aqueous Buffer Solutions

Figure 5-1 shows the dissolution profiles of taxol in water at various temperatures. As can be seen from these curves the concentration of taxol, at 37, 50, and 60° C, was substantially greater than that in 25° C. The dissolution rates in these media increased as temperature increased. This parallels the order observed for the equilibrium solubility of taxol in water at different temperature (Chapter 2). The dissolution behavior of taxol does not change considerably in different buffer solutions (Figure 5-2), suggesting that dissolution was essentially pH-independent at 25° C and taxol contains no groups that are ionizable in an acceptable pH range and therefore, pH adjustment did not produce a significant increase in solubility and dissolution of taxol ($P > 0.05$).

5.3.2 Effect of Bile salts on Dissolution of Taxol

Bile salts are the main active constituents in bile fluid which is of great interest in biopharmaceutics. These physiological surfactants have been shown to be an important part of the digestion and absorption of lipophilic compounds in the small intestine. Their physicochemical properties are similar to those of typical colloidal surfactants, including their ability to solubilize poorly water-soluble substances above the critical micelle concentration (CMC, Bates *et al.* 1966, Coello *et al.* 1996). Changes in the dissolution characteristics of drugs in the presence of bile salts depends on the physicochemical properties of the drug as well as the concentration of bile salt in the dissolution media (Bakatselou *et al.* 1991, Lippold *et al.* 1986). Figure 5-3 shows the dissolution behavior of taxol in water, and in 0.5 to 20 % w/v bile salt solutions at 37° C. As can be seen from these curves the concentration of taxol, at any time, was substantially greater than that in water. The dissolution rates in these media increased as concentration of bile salts increased. This parallels the order observed for the equilibrium solubility of taxol in water and in the presence of different concentration of bile salt (Chapter 2). The relative dissolution rates (Bates *et al.* 1966), that is, the amount of drug solubilized in the bile salts media divided by the amount of drug solubilized in water at the same time-interval is shown in table 5-1. At 2 hr relative dissolution rate of taxol in bile salt solution 20%w/v was 35-fold higher than in water. Table 5-2 shows the $t_{50\%}$ of dissolution of taxol (time required to reach 50% of solubility). The $t_{50\%}$ of taxol in bile salts solutions decreased as the concentration of bile salts increased. Solubility and dissolution rate of many drugs have been reported to be increased in the presence of bile salts. Generally, based on the Noyes-Whitney equation (Eq 1), the addition of surface active agents in the dissolution medium can be expected to alter the saturation solubility (C_s) of a drug via solubilization, or increases their surface area (A) via wetting effects or changes in the diffusivity of the compound (D) via convective diffusion or controlled reaction. The contribution of solubilization by bile salts to the dissolution rate of drugs has been studied extensively (Bates *et al.* 1966, Bakatselou *et al.* 1991, Lippold *et al.* 1986, Smidt *et al.* 1990, Rosoff *et al.* 1980, Miyazaki *et al.* 1981, Coello *et al.* 1996). Bile salts have shown a concentration-dependent micelle structure above the CMC. As shown in figure 5-4 the solubility of taxol increased non-linearly with the concentration of bile salts. This figure gives an apparent CMC of 3% (w/v) for bile salts in distilled water. Since the wetting effect of bile salts is of importance only at premicellar levels, it was concluded that in the studied concentration regions, although wetting and diffusivity effects of bile salts can be important in the improvement of the dissolution rate of taxol, the contribution of micellar solubilization of bile salts is a predominate factor in dissolution enhancement of taxol in bile salt solutions.

5.3.3 Effect of Cyclodextrins on the Dissolution of Taxol

Cyclodextrins are oligomers of glucose which are produced by enzymatic (cyclodextrin transglycosylase, CTG) degradation of starch. Cyclodextrins can substantially increase the aqueous solubility of pharmaceuticals. Based on the Noyes-Whitney equation for a poorly water-soluble compound like taxol, the saturation solubility term is one of the more important factors governing the rate of dissolution of the drug. Since the dissolution rate (dc/dt) is directly proportional to the solubility of a drug, any change in the nature of the dissolution medium which effectively increases C_s can, in theory, lead to an increase the rate of dissolution of drug. Figures 5-5 and 5-6 show the dissolution behavior of taxol in the dissolution media of different concentrations of HPβCD and γCD, respectively. As can be seen from these curves the concentration of taxol at any time is substantially greater than that in water. This parallels the order observed for the equilibrium solubility of taxol in water and in the presence of different concentrations of HPβCD and γCD (Chapter 2). The dissolution rates in these media increased as concentration of cyclodextrins increased. Table 1 shows the relative dissolution rates of taxol in HPβCD and γCD media at different time-intervals. At 2 hr the relative dissolution rate of taxol in HPβCD and γCD solutions (20%w/v) were 22- and 18-fold higher than in water, respectively. Table 5-2 shows the $t_{50\%}$ of dissolution of taxol. The $t_{50\%}$ of taxol in HPβCD and γCD solutions increased as the concentration of cyclodextrins increased. This rapid dissolution ($t_{50\%} < 0.3$ hr) at low cyclodextrin concentrations illustrated that, although taxol was dissolved very rapidly, its solubility was so low that maximum saturable solubility reached quickly. Therefore any improvement in solubility of taxol can increase the dissolution rate of taxol.

Solubility and dissolution rate of many drugs have been reported to be increased in the presence of HPβCD and γCD. Cyclodextrins have not shown any significant surface activity at concentrations up to 20%w/v. Therefore, it is concluded that the enhancement of dissolution rates of taxol in water by HPβCD and γCD could be due to the improvement of solubility of taxol through inclusion complexation with cyclodextrins. Similar mechanism for changes in the solubility and dissolution characteristics of some of the nonsteroidal anti-inflammatory drugs (Masuda *et al.* 1984, Tarimci and Celebi, 1988, Zecchi *et al.* 1988, and Pop *et al.* 1991), and silymarin (Arcari *et al.* 1992), and psoralen (Vincieri FF., *et al.* 1995) have been reported.

5.3.4 Effect of Nicotinamide on the Dissolution of Taxol

Nicotinamide is a nontoxic B vitamin (vitamin B_3) that has been shown to enhance the aqueous solubility and dissolution of many drugs through complexation (Truelove *et al.* 1984, Rasool *et al.*, 1990, Hussain *et al.*, 1992). The exact mechanism by which this compound forms complexes is not entirely clear, although it has been claimed to be mostly as the results of a *Pi*-donor *Pi*-acceptor interactions as mentioned in previous chapters. Naggar *et al.* (1980) have shown that nicotinamide can increase the dissolution of nitrofurantoin in either water or Sorensen's phosphate buffer (pH 5). Dissolution rate studies by El-Banna, *et al.* 1977, revealed that the fused nicotinamide/caffeine (50:50 and 70:30) solid dispersions exhibited faster dissolution rate (3.7-fold faster) than the pure drug.

Figure 5-7 shows the dissolution behavior of taxol in dissolution media as a function of nicotinamide concentration. As can be seen from these curves the concentration of taxol, at any time, is substantially greater than that in water. This parallels the order observed for the equilibrium solubility of taxol in water and in the presence of different concentration of nicotinamide (Chapter 2). The dissolution rates in these media increased as the concentration of nicotinamide increased. Table 1 shows the relative dissolution rates of taxol in the nicotinamide media at different time-intervals. At 2 hr relative dissolution rate of taxol in nicotinamide solutions (20%w/v) was 40-fold higher than in water, respectively. Table 5-2 shows the $t_{50\%}$ of dissolution of taxol. The $t_{50\%}$ of taxol in nicotinamide solutions increased as the concentration of nicotinamide increased. This rapid dissolution ($t_{50\%} < 0.3$ hr) at low nicotinamide concentrations illustrated that, although taxol was dissolved very rapidly, its solubility was so low that maximum saturable solubility reached quickly. Therefore any improvement in solubility of taxol can increase the dissolution rate of taxol.

The dissolution rate of taxol was enhanced by increasing the concentration of nicotinamide. Nicotinamide has shown some small surface activity at concentration above 5% w/v, also it was previously shown that the solubility of taxol can be dramatically improved through complexation with nicotinamide. Therefore, it was concluded that in the studied concentration regions, even though the surface activity of nicotinamide may improve wettability and diffusivity of this compound, the contribution of the solubilizing effect of nicotinamide can be a predominate factor in dissolution enhancement of taxol in nicotinamide solutions.

5.4 Conclusions

The effects of pH, temperature, bile salts, nicotinamide, γ-cyclodextrin, and hydroxypropyl-β-cyclodextrin on the dissolution of taxol have been investigated. dissolution of taxol at low cyclodextrin and nicotinamide concentration is rapid. This quick dissolution illustrates that taxol is dissolved very rapidly but its solubility is so low that maximum saturable solubility reached quickly and rate limiting step for poor availability is the poor solubility and not dissolution characteristics of taxol. Therefore, it seems that dissolution properties of taxol are crucial in its oral bioavailability and the rate and extent of oral absorption of taxol can be improved by using solubilizing agents. However, further studies are required to investigate the possible changes in other physicochemical and pharmacokinetic disposition properties of taxol in the presence of these solubilizing agents, and thereby, its toxicity and therapeutic effects under those conditions.

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Table 5-1 **Relative dissolution rate of taxol in water at 37° C in the presence of different solubilizing agents**

Medium	Time-interval (hr)		
	2	4	8
Bile salts 20% w/v	35	42	45
Nicotinamide 20% w/v	40	58	72
HPβCD 20% w/v	22	32	39
γCD 20% w/v	18	20	21

* Relative dissolution rates is the amount of a drug solubilized in each medium divided by the amount of the drug solubilized in water at the same time-interval.

Table 5-2 **$t_{50\%}$ (hr) of dissolution of taxol in water at 37° C in the presence of different solubilizing agents**

Concentration	Bile salts	Nicotinamide	HPβCD	γCD
0%	0.2	0.2	0.2	0.2
0.5%	2.7	0.3	0.3	0.3
1%	2.5	0.3	0.3	0.3
2%	1.2	0.3	0.4	0.3
5%	0.5	0.5	0.5	0.4
10%	-	1.6	1.2	0.5
20%	0.4	1.7	2.0	0.6

$t_{50\%}$: time required to reach 50% of solubility (hr)

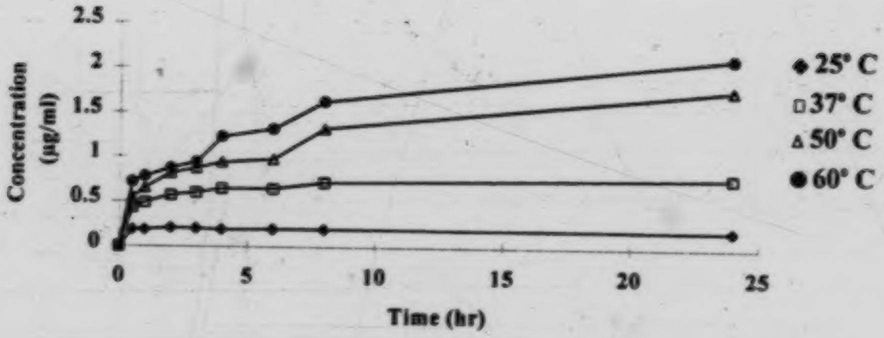

Figure 5-1 Dissolution of taxol in water at different temperatures. Data are presented as the mean of three experiments.

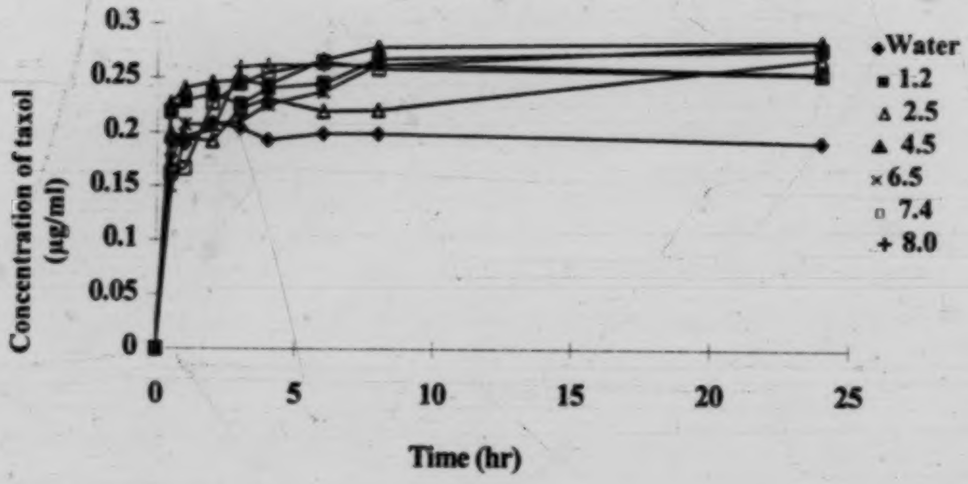

Figure 5-2 Dissolution of taxol in water at different pH at 25° C. Data are presented as the mean of three experiments.

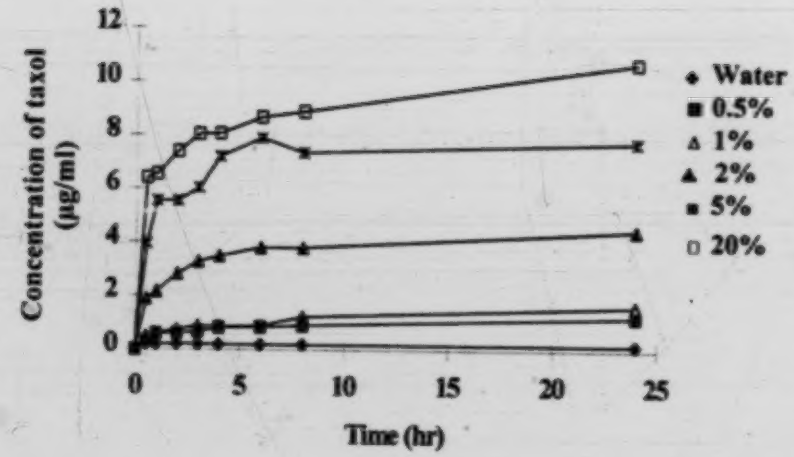

Figure 5-3 Dissolution of taxol in aqueous bile salt (Sodium Cholate:Sodium Deoxycholate, 50:50) solutions. Data are presented as the mean of three experiments.

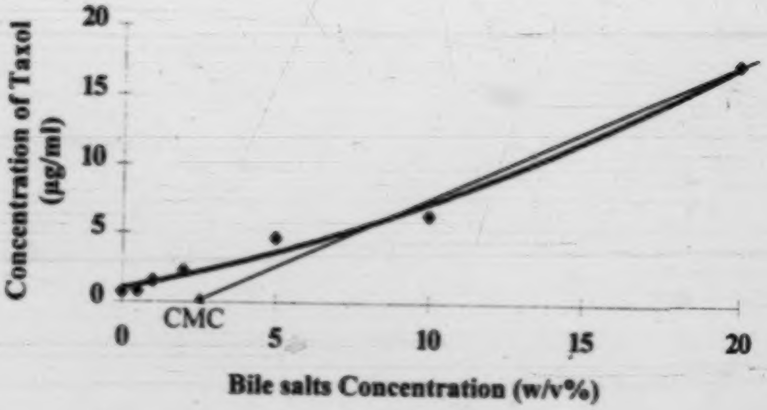

Figure 5-4 CMC of bile salts (Sodium Cholate:Sodium Deoxycholate, 50:50) in water. Data are presented as the mean of three experiments.

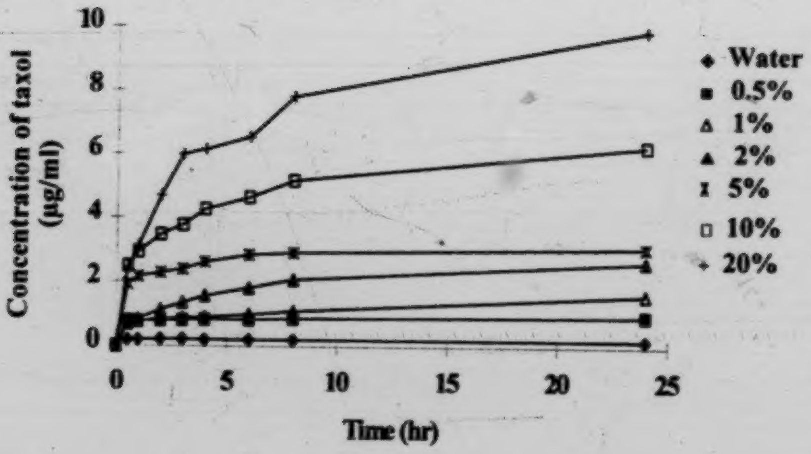

Figure 5-5 Dissolution of taxol in aqueous HPβCD solutions. Data are presented as the mean of three experiments.

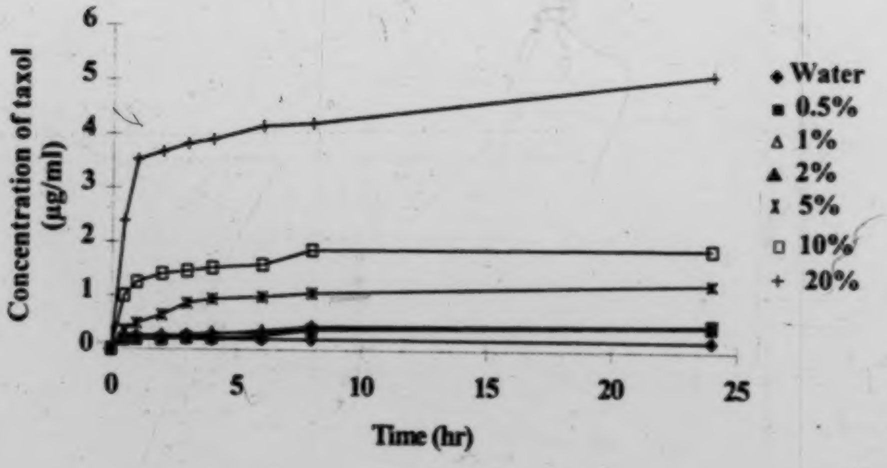

Figure 5-6 Dissolution of taxol in aqueous γCD solutions. Data are presented as the mean of three experiments.

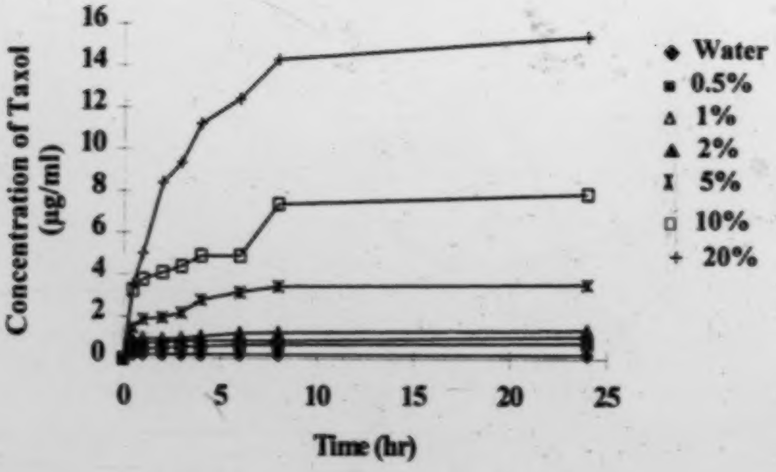

Figure 5-7 Dissolution of taxol in aqueous nicotinamide solutions. Data are presented as the mean of three experiments.

Chapter 6

6. The Cytotoxic Potential of Taxol in the Presence of Different Solubilizing Agents

6.1 Introduction

A major barrier to the study of the effects of chemicals on the antitumor response of mammalian cells *in vitro* and *in vivo* can be attributed to the poor water solubility of many of these compounds. Utilization of co-solvents, or solubilizing agents is an effective approach to overcoming this problem. However, it should be considered that these solubilizing agents and additives potentially have some intrinsic cytotoxicity which may change the cytotoxic activity as well as other physicopharmaceutical, pharmacokinetic and pharmacodynamic properties of the test compound. For example, Fjallskog *et al.*, (1994) have shown that when taxol is dissolved in ethanol, the effects on the corresponding doxorubicin-resistant sublines are significantly reduced compared with paclitaxel dissolved in ethanol:cremophor EL. Watts and Woodcock (1981) reported that ethanol and DMSO had no effect on the sensitizing efficiency and cytotoxicity of misonidazole. Sampedro *et al.* (1994), have reported that liposome entrapment can alter drug cellular uptake or may result in chemical modifications of the entrapped drug and thereby its pharmacodynamics and toxokinetics.

It seems that the cytotoxicity of most solubilizing agents is dose-dependent. For example dimethylacetamide, absolute ethanol and PEG 400 were evaluated as vehicles for solubilizing various hydrophobic compounds. Although dimethylacetamide is a very good co-solvent, even small amounts of this solvent are extremely cytotoxic to MCF-7 cells. Addition of absolute ethanol and PEG less than 0.1% to culture media is non-cytotoxic but at this concentration these solvents are unable to significantly solubilize many of the hydrophobic compounds (Denning and Webster, 1991). Substantial enhancement of cytotoxicity and subsequent increase in the life span of animals bearing the S-180 tumor after treatment with anticancer drugs containing Tween-80 as their vehicle has been reported (Menon *et al.* 1984). Therefore, in this study we evaluated the potential cytotoxicity effects of cyclodextrins, nicotinamide, PEG, bile salts, liposome, and cremophor EL, alone and in combination with taxol.

In general, in a cytotoxicity assay identical inocula of cells, such as KB cells, is tested for their response (growth or viability) to putative cytotoxic agents or conditions such as radiation. KB cells, an established line of human epidermoid carcinoma cells, have been used for cytotoxicity assays required in the development of new anticancer compounds since 1961 (Shoemaker *et al.* 1983). The KB cells have been proven to be stable, (as a substrate for cytotoxicity testing), in response to treatment over a considerable period of time. Cytotoxicity is usually tested by assaying the integrity of the cellular membrane, counting cells with either a hemocytometer or an electronic particle counter, detecting DNA content, or staining cell nuclei. Among these techniques, staining cell nuclei with crystal violet seems to be less laborious, less costly and extremely sensitive and rapid (Shoemaker *et al.* 1983).

6.2 Material and Methods

6.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA, and used as supplied. Cremophor, ß-cyclodextrin, α-cyclodextrin and hydroxypropyl-ß-cyclodextrin (molecular weight=1380) were obtained from Aldrich Chemical Company, Milwaukee, WI, USA, and were used without further purification. Nicotinamide and bile salts were supplied by Sigma Chemical Company, St. Louis. KB cells (ATCC CCL 17), CS-MEM (10% (Minimum Essential Medium, modified with Earles salt, and supplemented with 10% calf serum, 100 iu/ml penicillin G, and 100 µg/ml streptomycin),was supplied from Sigma Chemical Company, St. Louis. Water was deionized, Milli-Q (Millipore) purified. All other materials were reagent grade.

6.2.2 Preparation of Test Solutions

Solutions of solubilizing agents were prepared by dissolving bile salts, nicotinamide, γCD, HPβCD, ethanol, PEG, or cremophor:ethanol (50:50) in 10% CS-MEM to a final concentration of 20% (w/v or v/v). Liposome formulations with and without taxol were prepared as described earlier (chapter 3). Taxol was dissolved in DMSO to 40 μg/ml (stock solution). Adequate volume of the stock solution of taxol, solubilizing solutions, and 10% CS-MEM culture medium were mixed to study the cytotoxic potential of 1) each solubilizing agent, 2) a mixture of taxol (0.5 μg/ml) and solubilizing agent (10 % w/v or v/v), 3) taxol (0.025 μg/ml) in the presence of a fixed concentration of solubilizing agent (1.25% w/v or v/v), and 4) each solubilizing agent (10% w/v or v/v) in the presence of a fixed concentration of taxol (0.025 μg/ml).

6.2.3 Cell Culture Studies

KB cells were cultivated in Eagles minimum essential medium supplemented with 10% calf serum and incubated at 37° C in a humidified, 5% CO_2 atmosphere to prepare a cell stock solution. Cells were counted using a Neubauer hemocytometer and seeded in 96 well plates at 100 μl of 3 X 10^4 cells per ml and cultured for 1 day to allow the cells to adhere.

6.2.4 Toxicity Measurements

Test solutions were diluted 10, 100, 1000, 10,000 times with 10% CS-MEM. Then, 100 μl of the diluted test solutions were added to a separated well in a 96-well plate (all experiments were done in triplicate). Control wells were identical except that taxol and solubilizing agents were absent. Plates were cultured for three days. The cells were then fixed by adding 20 μl of 25% glutaraldehyde to the well-plates for 15 minutes. The unfixed cells (dead) were shaken out, and fixed cells were washed with water and dried, then stained in 100 μl of 0.05% crystal violet for 15 minutes followed by shaking out unbound dye. The wells were washed with water, dried then bound stain was eluated with 100 μl of 0.05 M NaH_2PO_4/ethanol (1:1v/v) and optical density were measured (OD_{540}) on a multiscan spectrophotometer, and the TD_{50} values were calculated using the formula:

$$TD_{50} = \{[(0.5 \times A) - H] \div [L - (0.5 \times A)]\} \times (C_H - C_L)$$

where A is 50% of the average ODS of control wells, H is the first ODS value greater than A, L is the first ODS value less than A, C_H is the concentration of compound in the well with value H, and C_L is the concentration of compound in well with value L. If there were several wells at each concentration, A, H, and L were the averages of the values.

6.2.5 Statistical Evaluation

Statistical evaluations were performed on all data using One-way ANOVA. Where a significant F-value was obtained, the Bonferroni method was used for pairwise multiple comparisons, the level of significance chosen for all statistical analyses being $\alpha = 0.05$. The results were expressed as means ± standard deviation (STDEV).

6.3 Results and Discussion

The cytotoxic potential of taxol was evaluated *in vitro* against KB cells and in the presence of different solubilizing agents to assess the role of vehicles in overall cytotoxicity of this compound. The cytotoxic potential of different solubilizing agents is shown in figure 6-1. Among solubilizing systems tested, liposome (15 mM, DPPC:CHL 2:1) showed the lowest cytotoxicity followed by γCD and HPβCD (not significantly different than water, liposomes and γCD, ($P < 0.05$). Cremophor EL showed the highest cytotoxicity for KB cells followed by bile salts, ethanol, nicotinamide and PEG ($P < 0.05$). Figure 6-2 compares the cytotoxicity of taxol in different formulations. Cell toxicity of taxol in the formulations containing nicotinamide, liposomes, and PEG decreased (compared to water) while formulations containing bile salts, ethanol and cremophor EL increased the cytotoxic effect of taxol (compared to water). HPβCD and γCD formulations did not change the cytotoxicity of taxol (compared to water, figure 6-2). In the presence of a fixed concentration of taxol (0.025 μg/ml) all cells were killed after 3 days and a ranged of concentrations of solubilizing agents (1×10^{-4}% to 10%) did not make any difference changes in the cytotoxicity of taxol. The TD_{50} of taxol in the presence of high and fixed concentration (1.25 w/v%) of γCD did not change significantly (figure 6-3), while in the presence of 1.25 w/v or v/v% bile salts, nicotinamide, HPβCD, PEG, nicotinamide, or cremophor the TD_{50} of taxol decreased.

These data reveal that bile salt and cremophor at high and even low concentration can increase the cytotoxicity of taxol. Surface active agents have been shown to be capable of changing the regulation of cell membrane, enhancing the permeation of drugs to the cells, and decreasing the efflux of drugs from cells. These properties may explain the cytotoxic effect of bile salts and cremophor. Increase in the cell toxicity of taxol in the presence of high concentrations of HPßCD and nicotinamide is an interesting phenomenon. It has been reported that the presence of high concentration of HPßCD and nicotinamide at the site of absorption can change the permeation of drug through the cell membrane possibly by changing the partitioning of drug or complexation with the phospholipids of cell membrane, and thereby, interrupting membrane function (Vollmer *et al.* 1994, Muller *et al.*, 1991, Shao *et al.*, 1992, and 1994). Low concentrations of HPβCD did not significantly change the cytotoxicity of taxol against KB cell line. On the other hand, nicotinamide decreased the cytotoxicity of taxol. At this point the exact mechanism/(s) involved are unknown, however, this phenomenon may be unrelated to changes in the physicochemical properties of taxol (we have already in chapters 2, 3, and 4 shown that the complexation of HPßCD and nicotinamide with taxol is concentration-dependent but at low concentration there is no significant change in the physicochemical properties of taxol). Although it is well established that nicotinamide is an active cell component and may increase the viability of cells at low concentration, further studies are needed to clarify the mechanisms involved.

6.4 Conclusions

Among solubilizing systems tested, liposome, γCD, and HPβCD showed the lowest cytotoxicity and cremophor EL, bile salts, ethanol, nicotinamide and PEG showed the highest cytotoxicity for KB cells. Presence of HPβCD, nicotinamide, bile salts, ethanol, and cremophor EL increased the cytotoxicity of taxol by increasing the permeation of taxol and/or by synergism effect. Therefore, it is advisable to consider the potential cytotoxicity effects of solubilizing agents alone and in combination with taxol in the development and evaluation of new taxol formulations.

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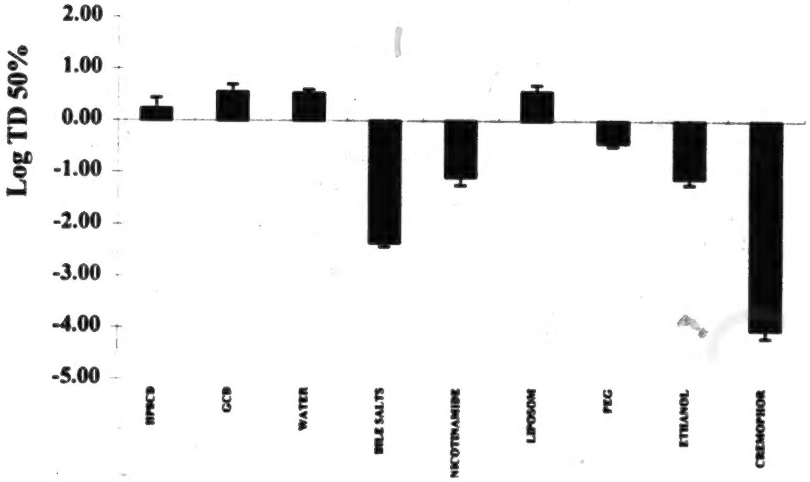

Figure 6-1 **The cell toxicity of different solubilizing agents. Data are presented as the mean ±STDEV (n=3).**

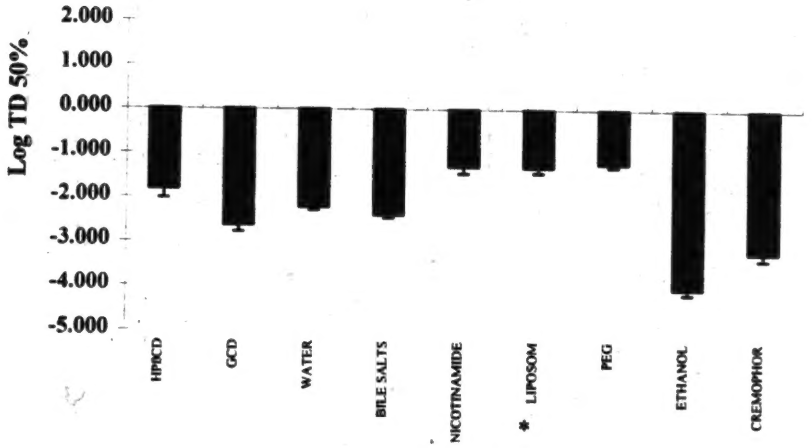

Figure 6-2 Cell toxicity of taxol (0.5 µg/ml) in 10% (w/v) solution of different solubilizing agents. Data are presented as the mean ±STDEV (n=3). * 3% total lipid

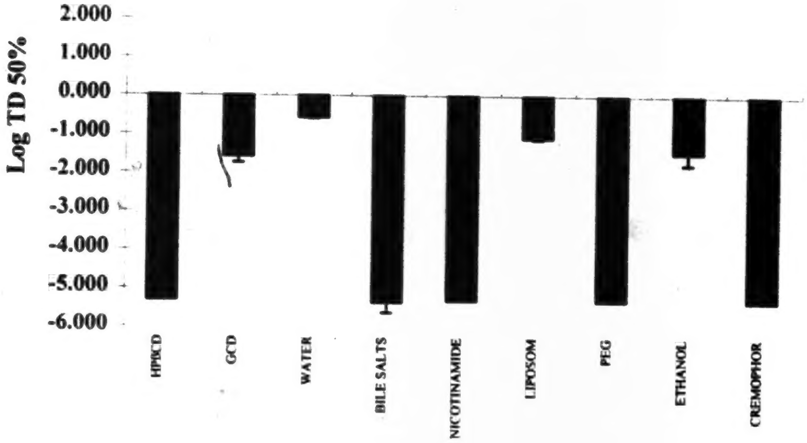

Figure 6-3 Cell toxicity of taxol in the presence of a fixed concentration of solubilizing agent. Data are presented as the mean ±STDEV (n=3).

Chapter 7

7. Effect of Hydroxypropyl-β-Cyclodextrin and Nicotinamide on the Oral Absorption of Taxol.

7.1 Introduction

Taxol has a unique mechanism of action, resulting in stabilization of tubulin polymerization resulting in arrest in mitosis and cell death. *In vivo* studies have shown a significant impairment in the oral absorption of taxol in rat. Since taxol is poorly water-soluble, the investigation of its intestinal absorption characteristics after administration of solutions and suspension was needed before the development of oral dosage forms. Scientific, financial, and ethical considerations regarding the use of whole animal for screening the gastrointestinal absorption potential of taxol, have led to the use everted rat intestine technique to mimic the *in vivo* absorption characteristics of taxol (Hillgren *et al.* 1995). Examination of absorptive processes has been accomplished using everted rat intestinal tissue. Since nicotinamide and hydroxypropyl-β-cyclodextrin has already been shown to increase the solubility and dissolution rate of taxol and many other drugs (Tous, *et al.* 1992, Schipper et 1993, Hozbor *et al.* 1994, Reer *et al.* 1994, Vollmer *et al.* 1994, Matsubara *et al.* 1995, and Hovgaard *et al.* 1995, the intestinal absorption of taxol using the everted rat intestinal was investigated. The study included investigations of the influence of intestinal site, taxol concentration, taxol formulation (suspension vs solution), and presence of solubilizing agents (nicotinamide, and hydroxypropyl-β-cyclodextrin) on the absorption of taxol.

7.2 Materials and Methods

7.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA, and used as supplied. The following cyclodextrins were obtained from Aldrich Chemical Company, Milwaukee, WI, USA, and were used without further purification: α-cyclodextrin, ß-cyclodextrin, hydroxypropyl-ß-cyclodextrin (molecular weight=1380). Nicotinamide was supplied by Sigma Chemical Company, St. Louis, MO, USA. Gamma-cyclodextrin was obtained from American Maize-Products company, Hammond, IN, USA. Water was deionized, Milli-Q (Millipore) purified. All other materials were reagent grade.

7.2.2 Everted Rat Intestine Studies

Sprague-Dawley rats (350-400 g) were anaesthetized with ether and the intestines were exposed through a vertical midline incision. The jejunum, ileum and colon segments were removed. The excised intestines were immediately placed in ice-cold oxygenated buffer (95% O_2/5% CO_2-saturated Krebs-Henseleit, pH 7.4) containing 2 g/L of dextrose. Each part was then everted over a glass rod, placed on chilled glass surface, and covered with cold 95% O_2/5% CO_2-saturated Krebs-Henseleit solution. Segments (15 cm) were cut, one end was tied off, and the sac was filled with 15 ml of pre-equilibrated 37°C, drug-free, 95% O_2/5% CO_2-saturated Krebs-Henseleit solution. To initiate uptake studies, sacs were incubated in a jacketed, glass organ bath containing uptake solutions (either 30 ml of 95% O_2/5% CO_2-saturated Krebs-Henseleit or solutions of HPβCD 20% (w/v) or nicotinamide 20% (w/v) in 5% O_2/5% CO_2-saturated Krebs-Henseleit solution). After 30 min, taxol was added to the uptake solution as a suspension (30 µg/ml) or solution (10-60 µg/ml). Sampling was done at appropriate times from uptake solutions (mucosal site) and inside the everted intestine (serosal site). Contents of the organ bath was maintained at 37° C and continually oxygenated (95% oxygen-5% carbon dioxide) during the experiment. Aliquots (0.4 ml) of the solution were withdrawn at appropriate time intervals and processed as directed in the assay of taxol. The resultant loss in volume was compensated by adding the Krebs-Henseleit solution maintained at the same temperature. In order to compensate for the previously-removed samples, a cumulative correction was made using the following formula:

$$C_n = C'_n + (V_s/V_d) \times C_{n-1}$$

where C_n is the expected concentration of the n_{th} sample if previous samples had not been removed; C'n is the measured concentration; C_{n-1} is the expected concentration in the previous sample, V_s is the sample volume, and V_d is the original solution volume in serosal or mucosal site. The disappearance rate constant (k_{dis}) was calculated from the logarithm of the transferable concentration of taxol in the mucosal site against time by linear regression. All measurements were made in triplicate.

7.2.3 Methods of Analysis of Samples

Samples were analyzed for taxol using a Waters HPLC system (Lc module 1) consisting of a model 600E pump, a model 715 auto-injector, and a model 486 UV detector, with a partisil ODS (phenomenex ® 100 mm, 4.6 mm ID, 5 µm particle size) column protected by a partisil ODS (phenomenex ® 30 mm, 4.6 mm ID, 5 µm particle size) guard column, using a mobile phase of 40% acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.0) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm. Samples of 1 ml plus 0.1 ml of internal standard (fenoprofen 3.5 µg/ml in acetonitrile 47% in water were extracted with 3.5 ml of *t*-butyl methyl ether and vortex-mixed for 1 min. The mixture was then centrifuged for 10 min at 1100 g after which 3 ml of the organic layer were removed and evaporated to dryness under vacuum at low temperature. The residue was reconstituted in 0.2 ml of 47% aqueous acetonitrile and injected (50 µl) into the HPLC system. Standard solutions of taxol were made when appropriate, extracted and injected before, during, and after a series of samples was injected.

7.3 Results and Discussion

7.3.1 Absorption of Taxol

As shown in figure 7-1, taxol in cremophor EL formulation (25 µg/ml) disappeared from the mucosal side of everted rat intestine with apparent first-order kinetics. The disappearance rate constant (k_{dis}) was calculated from the logarithm of the transferable concentration of taxol on the mucosal side against time by linear regression and the results are given in Table 7-1. No significant region-dependent variations in the intestinal absorption of taxol were detected (Figure 7-2). Although the upper site (jejunum) of the small intestine tends to have the larger disappearance rate constant, however, there were no statistically significant differences ($P > 0.05$) in the percent of taxol remaining at the mucosal site at each time point between the parts of the intestine. Taxol was not detected in the serosal site of the intestine from the suspension formulation and the remaining taxol in the mucosal site was also nearly constant. Therefore, absorption of taxol to the everted rat gut from suspension seemed to be negligible, probably because of the lack of solubility of taxol (solubility of taxol in Krebs solution is about 1 μg/ml at 37°C). Figure 7-3 reveals the relationship between concentration of taxol in the serosal and mucosal site of rat everted intestine after administration of cremophor formulation with different concentration (5-60 μg/ml). Increasing the soluble taxol concentration in the mucosal site from 5 to 60 μg/ml leading to an increase in the concentration of taxol in the serosal site of intestine from 0.5 μg/ml to 1.6 μg/ml. Comparing the percentage of drug which passed through the intestine wall after 4 hr incubation with different doses of taxol showed that there was a nonlinearity in oral absorption of taxol. Therefore, involvement of a saturable mechanism in the GI absorption of taxol is possible. The rate of taxol absorption was low and dependent on the concentration of soluble form of taxol at the site of absorption.

7.3.2 Effect of HPβCD on Absorption

The effects of chemically-modified cyclodextrins on the cellular nasal, transdermal, corneal, and oral absorption of drugs have been investigated using different *in vitro* and *in vivo* models. The absorption enhancer effects of these cyclodextrins for mucosal transport of poorly absorbed drugs have been evaluated by different techniques including bioavailability studies, staining of the cytoplasm, determination of the mitochondrial dehydrogenase activity as well as hemolytic activity and transport enhancement of the macromolecular pore marker polyethylene glycol 4000 (PEG-4000).

Figure 7-4 shows the effects of HPβCD on the amount of taxol transported across the everted rat intestine. The amount of taxol that passed through the intestine containing 20% w/v HPβCD formulation was 2.7 fold more than from cremophor EL formulation.

Previously it was shown that HPβCD formed inclusion complexes with taxol which may improve solubility, dissolution, and also participate in the protection of the drug against degradation. Additionally, HPβCD is shown to increase the permeability of mucosal membranes.

The mode of action of membrane penetration enhancement of different cyclodextrins varies and depends on the structure of cyclodextrin and the membrane. Vollmer *et al.* (1994) showed that, based on the results of DSC and permeability experiments, the thermal profile of human stratum corneum is practically unchanged after treatment with HPβCD, but treatment with dimethyl-beta-cyclodextrin (DMβCD) revealed an interaction of DMβCD with the protein and lipid fractions. Therefore, they concluded that DMβCD acts as a transdermal absorption enhancer by changing the stratum corneum barrier, whereas HPβCD influenced the partitioning behavior of the drug in the skin. Also treating Caco-2 monolayers with DMβCD decreased the transepithelial electrical resistance, which is an indication that the tight junctions of mucosal membranes can be disrupted by cyclodextrins (Hovgaard *et al.* 1995). Also, it has been shown that in the presence of cyclodextrin, Bordetella Pertussis cells can enhance the levels of the intracellular form of adenylate cyclase (200 kDa) and two other extracellular proteins, pertussis toxin and filamentous hemagglutinin. It has been suggested that the presence of cyclodextrin can destabilize the outer membrane of these cells which motivate the release of those proteins. It has also been shown that cyclodextrins only change the outer membrane permeability and do not modify the fluidity of their cell membrane (Hozbor *et al.*, 1994).

The permeability enhancer effect of cyclodextrins on the cell membrane has been reported to be reversible after removing the cyclodextrins from media. However, membrane absorption is not completely returned to normal levels immediately (Schipper *et al.* 1993). It has been reported that the barrier function of the mucosal membrane restored itself within 24 hrs after termination of exposure to considerable concentration of cyclodextrins. Scanning electron microscopic observations revealed that DMβCD induced no remarkable changes to the surface morphology of the nasal mucosa at a minimal concentration necessary to achieve substantial absorption enhancement (Matsubara *et al.*, 1995). These results suggest that cyclodextrins may improve the bioavailability of drugs and are well-tolerated by the mucosal membrane and biological systems.

Results have shown that among cyclodextrins, DMβCD has been the most effective in improving the rate and extent of the nasal, transdermal and oral bioavailability of many drugs and HPβCD has revealed the most favorable toxicological properties (Reer *et al.* 1994).

7.3.3 Effect of Nicotinamide on Absorption

Nicotinamide is a nontoxic B vitamin (vitamin B_3) and has been shown to make a *Pi*-donor *Pi*-acceptor interaction with many compounds. The results of this interaction are changes in some of physicochemical parameters of a compound such as solubility, dissolution and stability. Previously it was shown that nicotinamide formed complexes with taxol which improved its solubility and dissolution and decreased its in water. Figure 7-5 shows the effects of nicotinamide on the amount of taxol transported across the everted rat gut. The amount of taxol leaving the mucosal site of the everted rat intestine containing 20% w/v nicotinamide formulation was 4.5 fold more than that of cremophor EL formulation. The mode of action of membrane penetration enhancement of nicotinamide is not clear. But it has been reported that nicotinamide is a cholesterol-lowering agent (Sugiyama K *et al.*, 1989),which dilates the blood vessels, improves tumor oxygenation (Chaplin *et al.* 1991), radiosensitises a wide variety of mouse tumors (Ono *et al.* 1993), decreases flow resistance in tumors (Lee *et al.* 1992), increases the cardiac output distribution (COD) of carbogen to the mouse carcinoma (growing in skin, muscle or the gut wall), and increases the regeneration response of the jejunum and the bone marrow following irradiation (Hirst *et al.* 1993). All these reports suggest that nicotinamide should somehow change the function of cell membranes. It is believe that these reports indicate that nicotinamide exerts multiple effects on cell membranes and that the extraction of a membrane component (cholesterol) is one of the effects, and opening of the tight junctions could be another possibility. These hypotheses must be studied in the future. Nicotinamide is currently under trial for the prevention of insulin-dependent diabetes mellitus after success in the NOD mouse (Stratford MR., *et al.* 1994).

7.4 Conclusions

The absorption of taxol by the rat everted intestine wall is low and not region-selective. The involvement of a saturable mechanism in the GI absorption of taxol is one of the possibilities for nonlinearity in oral absorption of taxol. An improvement in the absorption of taxol may be achieved by a combination effects such as improving taxol solubility and absorption enhancement by selected agents. These results suggest that cyclodextrins and nicotinamide may improve the bioavailability of taxol and should be well-tolerated by the mucosal membranes of biological systems.

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Table 7-1 The disappearance rate constant (k_{dis}) of the transferable concentration of taxol at the mucosal side of different parts of everted rat intestine. Data are presented as the mean ±STDEV (n=3).

Concentration of taxol in mucosal site (µg/ml)	disappearance rate constant (k_{dis}) (hr^{-1})		
	jejunum	ileum	colon
60	0.653 ± 0.231	0.359 ± 0.196	0.371 ± 0.186
25	0.791 ± 0.148	0.681 ± 0.223	0.309 ± 0.174
10	0.353 ± 0.081	0.59 ± 0.292	0.309 ± 0.169
5	0.1655 ± 0.0645	0.333 ± 0.235	0.167 ± 0.071

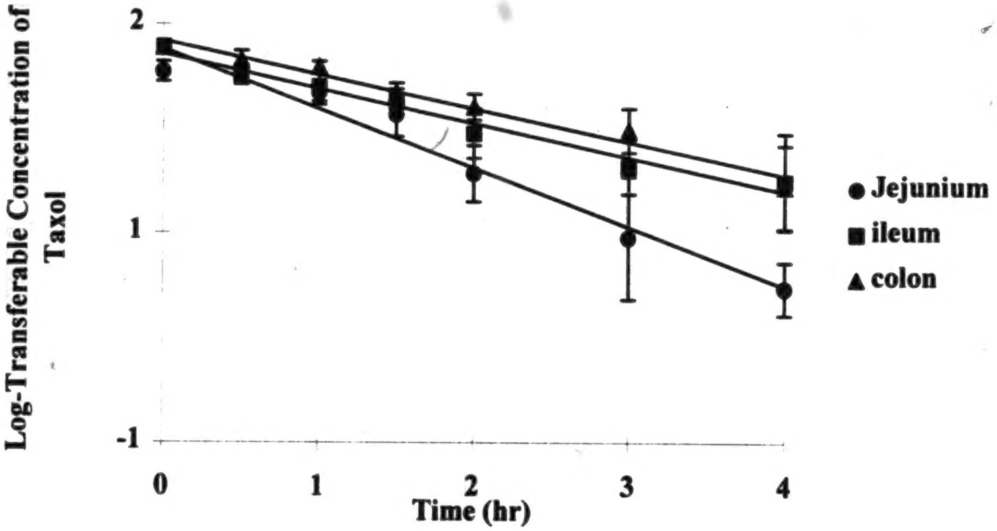

Figure 7-1 Plot of the Log-transferable concentration versus time of taxol across everted rat intestine. Data are presented as the mean ±SEM (n=3).

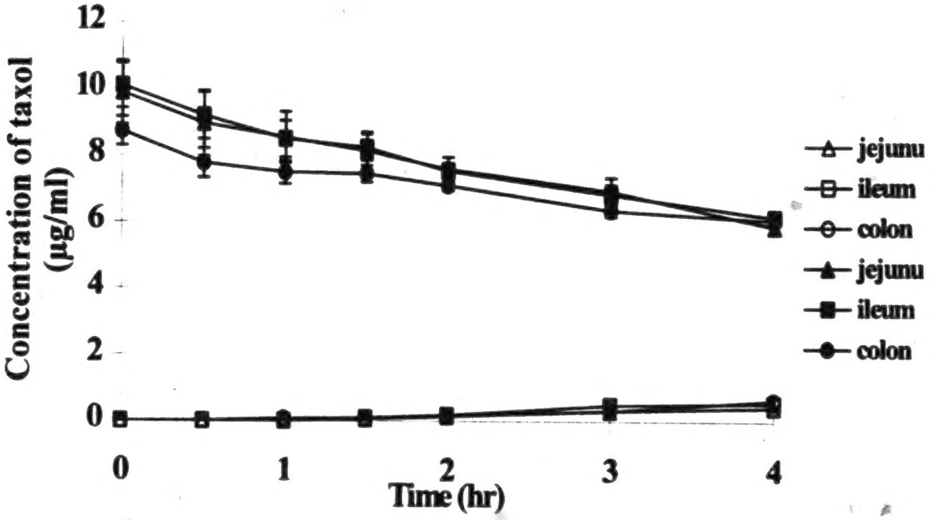

Figure 7-2 Concentration of taxol versus time at mucosal (●, ▲, and ■) and serosal sites(○, Δ, and □) of the different parts of the rat intestine using cremophor formulation (taxol 10 μg/ml). Data are presented as the mean ±SEM (n=3).

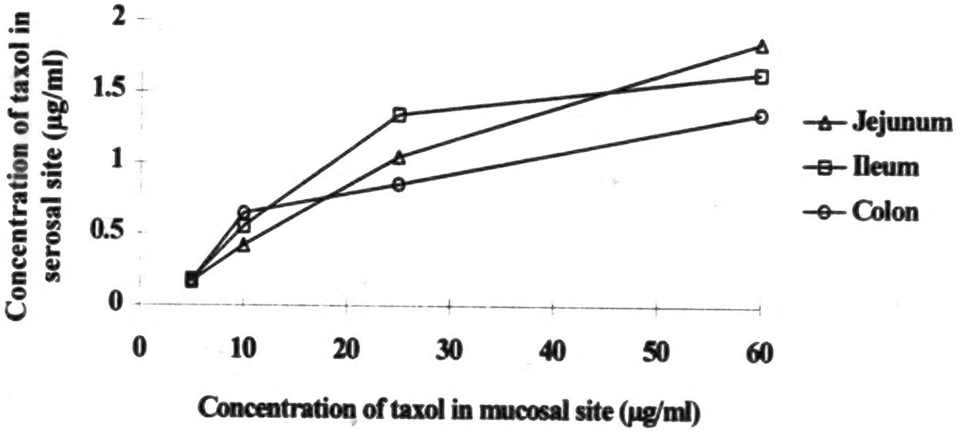

Figure 7-3 Relationship between concentration of taxol in mucosal (at t=0) and serosal site (at t=4 hr).

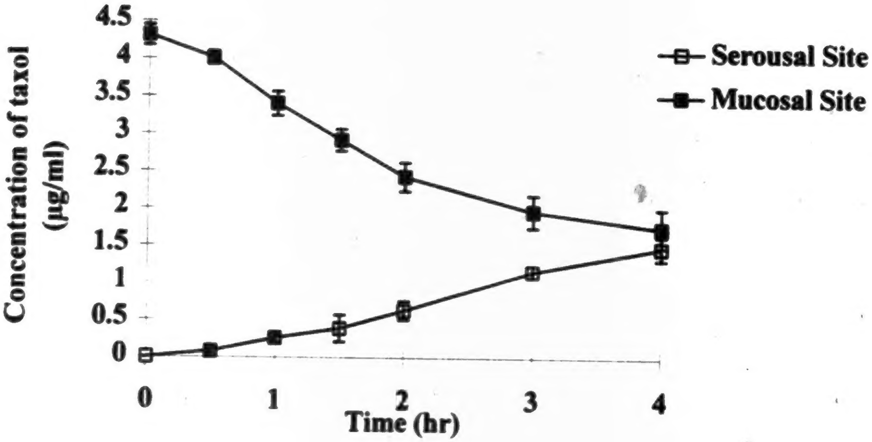

Figure 7-4 Concentration of taxol versus time at mucosal and serosal sites of rat jejunum using a HPβCD formulation (taxol 10 µg/ml). Data are presented as the mean ±SEM (n=3).

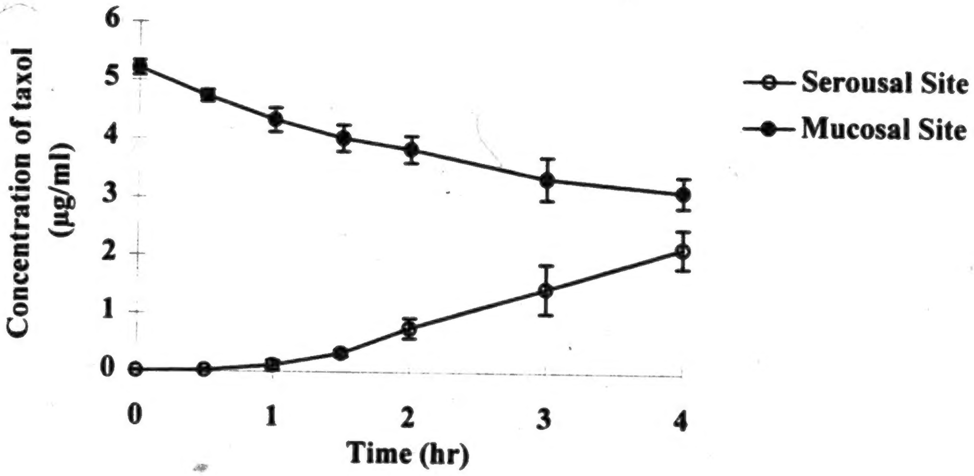

Figure 7-5 Concentration of taxol versus time at mucosal and serosal sites of rat jejunum using a using nicotinamide formulation (taxol 10 µg/ml). Data are presented as the mean ±SEM (n=3).

Chapter 8

8. Improving Quantitative Determination of Taxol in Biological and non-Biological Samples

8.1 Introduction

Taxol is a complex diterpenoid with a novel mechanism of action and impressive clinical activity. This compound is one of the more intriguing new anticancer drugs to enter the clinical arena in many years. Taxol was discovered in 1971, but due to insufficiency of preclinical pharmacological studies on that its clinical application was not approved by FDA until 1992. The lack of preclinical pharmacological studies with taxol was due largely to the agent's unique structure, specially its extreme insolubility in water and the insensitivity of standard analytical methods (Koller *et al.* 1994). Although several methods have been published before clinical trials began, they were generally time consuming and insensitive and therefore, were not suitable for use in large clinical investigations. The only pharmacological information available before clinical trials began was achieved with biochemical assay with sensitivity of 0.1 μmol/L (Hamel *et al.*, 1982). Several reverse phase, high performance liquid chromatographic (HPLC) methods were subsequently developed during early phase I investigations to measure taxol concentrations in biological samples. (Longnecker *et al.*, 1986, Grem *et al.* 1987, Brown *et al.* 1991, Wall *et al.* 1992). These HPLC assays, using similar extraction and analytical methods, permitted assessment of the pharmacokinetic behavior of taxol on many administration schedules. However, the variable extraction efficiencies, limits of sensitivity and other performance characteristics of the HPLC assay have generally rendered them unreliable, especially for the pharmacological monitoring of patients receiving lower doses of taxol infused over relatively longer periods ((Koller *et al.* 1994). A modified HPLC assay that uses a substantially longer analytical column appears to have increased sensitivity compared with earlier procedures (Rizzo *et al.*, 1990 and Wall *et al.*, 1992). Another, highly sensitive, HPLC assay with a lower limit of detection of 6 ng/ml (7 nmol/L) in plasma and 8 ng/ml (9 nmol/L) in urine has been reported (Beijine. 1993). This method consists of a selective solid phase extraction with a Bond Elut Cyano column followed by reverse phase HPLC with ultraviolet detection of 227 nm. Various immunological assays with sensitivity of 0.3 nmol/L have also been developed, including a rapid and sensitive indirect competitive inhibition enzyme immunoassay (CIEIA) and competitive enzyme linked immunosorbent assays (ELISA), primarily for measuring concentrations of taxanes in plant extracts (Grothaus *et al.*, 1992a,b; Leu *et al.*, 1993). Recently several different HPLC methods have been published (Mase *et al.*, 1994, Huizing *et al.* 1995, Song *et al.* 1995) However, these methods need large volume of biological samples and involve lengthy extraction procedures and consequently, are not very suitable for mass analysis, and preformulation studies of taxol. In this study we developed a method which is rapid and has enough sensitivity to follow concentrations of taxol in its preformulation studies.

8.2 Materials and Methods

8.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA, and used as supplied. The following cyclodextrins were obtained from Aldrich Chemical Company, Milwaukee, WI, USA, and were used without further purification: α-cyclodextrin, ß-cyclodextrin, hydroxypropyl-ß-cyclodextrin (molecular weight=1380). Nicotinamide was supplied by Sigma Chemical Company, St. Louis, MO, USA. Gamma-cyclodextrin was obtained from American Maize-Products company, Hammond, IN, USA. Water was deionized, Milli-Q (Millipore) purified. All other materials were reagent grade.

8.2.2 Chromatography

A Waters HPLC (Waters Lc module 1) consisting of a Model 600E pump, a 715 auto-injector, and a 486 multi-wavelength UV detector (waters, Mississauga, Canada) was operated at ambient temperature. The column was a partisil ODS (phenomenex ® 100 mm, 4.6 mm ID, 5 µm particle size) protected by a partisil ODS (phenomenex ® 30 mm, 4.6 mm ID, 5 µm particle size) guard column. The mobile phase consisted of 40%

acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.0) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm.

8.2.3 Extraction Procedure

8.2.3.1 Biological Samples

To 0.1 ml of plasma, 0.1 ml of urine, or 0.025 ml of bile, was added 0.1 ml of internal standard (fenoprofen 3.5 µg/ml in acetonitrile 47% in water). To this was added 1 ml of acetonitrile of HPLC-grade, to precipitate proteins. This mixture was mixed vigorously with Vortex Genie 2 mixer (Fisher Scientific, Edmonton, Canada), for 1 min then centrifuged at 3000 rpm on a Dynac II centrifuge (Becton Dickinson, Parsippany, NJ, U.S.A.), for 5 min. The top layer was transferred to a clean tube and was evaporated to dryness by using a SVC100H Savant Speed Vac Concentrator and Refrigerated Condensation Trap (Emerston Instruments, Scarborough, Canada). To the evaporated residue was added 200 µl of acetonitrile 50% followed by vortexing for 1 min. 50 µl of reconstituted sample was injected into the HPLC system. Standard solutions of taxol were made when appropriate, extracted and injected before, during, and after a series of samples were injected.

8.2.3.2 Non-Biological Samples

To 0.1-1 ml of filtered samples (0.45 µm) from solubility, dissolution, or stability studies, was added 0.1 ml of internal standard (fenoprofen 3.5 µg/ml in acetonitrile 47% in water). This mixture was mixed vigorously for 1 min and then was evaporated to dryness. To the evaporated residue was added 200 µl of acetonitrile 50% followed by vortexing for 1 min. 50 µl of reconstituted sample was injected into the HPLC system. Standard solutions of taxol were made when appropriate, extracted and injected before, during, and after a series of samples were injected.

8.2.3.3 Extraction Efficiency

For biological samples, an acetonitrile solution of taxol was evaporated to dryness. These samples (N =4) were then reconstituted to give final concentrations of 0.5 µg/ml and 5 µg/ml in plasma, urine, or bile. The solutions were extracted (precipitation) with 1 ml acetonitrile and exactly 1 ml of the supernatant was taken out. The supernatant was evaporated and the peak area ratios of taxol extracted versus unextracted equivalent concentrations of taxol were compared under identical chromatographic conditions.

8.2.4 Standard Curves.

8.2.4.1 Non-biological

8.2.4.1.1 Pure

Taxol, 1mg, was dissolved in 5 ml acetonitrile and made up to 10 ml with water. A 1-ml aliquot of this solution was further diluted to 10 or 100 ml with acetonitrile 50% to produce concentrations of 10 and 1 µg/ml, respectively. Different aliquots of the above solutions were added to acetonitrile 50% to final concentrations of 0.05 to 50 µg/ml (12 points). The standard samples were then treated in the same manner as described above for non-biological samples.

8.2.4.1.2 *In vitro* Samples

Taxol, 10 mg, was dissolved in 0.5 ml cremophor plus 0.5 ml absolute ethanol and made up to 10 ml with water. A 1-ml aliquot of this solution was further diluted to 10 or 100 ml with water to produce concentrations of 100 and 10 µg/ml respectively. Different aliquots of the above solutions were added to PEG, ethanol, cyclodextrins (αCD, βCD, γCD, and Hydroxypropyl-βCD), citrate buffers, phosphate buffers, nicotinamide, and bile salts solutions to final concentrations of 0.05 to 50 µg/ml (6 points). The standard samples were then treated in the same manner as described above for non-biological samples.

8.2.4.1.3 Biological Samples

Taxol, 10 mg, was dissolved in 0.5 ml cremophor plus 0.5 ml absolute ethanol and made up to 10 ml with water. A 1-ml aliquot of this solution was further diluted to 10 or 100 ml with water to produce concentrations of 100 and 10 μg/ml, respectively. To 0.1 ml of plasma, urine, or bile were added different aliquot of the above solutions to produce final concentrations of 0.1 to 10 μg/ml (10 points). The standard samples were then treated in the same manner as described above for biological samples.

8.2.5 Accuracy and Precision

To determine accuracy, taxol was added to drug free biological and non-biological samples and its concentration was calculated using the standard curves. The difference between the mean estimated and the mean added concentrations was taken as the accuracy of the method. Precision was estimated by determining the inter-assay coefficient of variation. The intraday (N= 4) and interday (N = 6) variations of the assay were examined for solutions containing 0.1, 0.5, 1, 5, and 20 μg/ml.

8.3 Results and Discussion

This HPLC method which employed direct injection of samples after precipitation of proteins and salts, offers a sensitive and convenient technique for measurement of taxol in biological and non-biological samples. Under the chromatographic conditions stated, virtual baseline, separation of taxol and internal standard was achieved (resolution, R> 2). Peaks representing the fenoprofen and taxol appeared 5.2± 0.2 and 7.4 ±0.2 min after injection, respectively (Figure 8-1). The order of elution of drugs was verified by subjecting individual drugs (fenoprofen or taxol) to the same procedure as the biological or non-biological samples. There were no interfering peaks from samples containing buffers, PEG, ethanol, cremophor, cyclodextrins, or normal component of plasma, bile, or urine.

Excellent linear relationships ($r^2 > 0.997$) were found between the taxol/fenoprofen peak-area ratios and the concentrations, in both biological and non-biological samples (Figures 8-2-a and 8-2-b). Regression lines through the data points were platted. The extraction efficiency of taxol in plasma, urine, and bile was greater than 92%. The intra- and interday variation of the assay was in the range of 1 to 5%. Using a signal-to-noise ratio of 4:1, the limit of reliable quantitation was set at 0.05 and 0.1 μg/ml for non-biological and biological samples, respectively, which encompasses the concentration observed in our *in vitro* and *in vivo* studies. The assay was found to be suitable for determination of taxol in rat plasma, following the ip administration of 33 mg/kg of dose of taxol, or solubility, stability, and dissolution studies of taxol.

Typical chromatograms obtained from extracted plasma samples are illustrated in figures 8-3-a and 8-3-b. Figure 8-3-a shows a representative chromatogram of a processed plasma blank. This chromatogram indicates that no endogenous compounds exist at the retention times of taxol or the internal standard. Figure 8-3-b is a chromatogram amplified to the same degree as the blank showing the limit of quantification (0.1 μg/ml). Figure 8-4-a is a chromatogram of a processed blood blank. This chromatogram indicates that, there are some endogenous compounds exist in the chromatogram but not at retention times of taxol or internal standard. The chromatogram of blood sample spiked with 10 μg/ml of taxol, is shown in figure 8-4-b. The chromatograms of plasma samples obtained from a rat before and after 6 hr of administration of a single 33 mg/kg ip dose of taxol are illustrated in figures 8-5-a and 8-5-b these chromatograms shows the suitability of this method for studying the pharmacokinetics of taxol in rats. The typical chromatograms obtained from non-biological samples in cyclodextrin (αCD, βCD, γCD, HPβCD), PEG, cremophor, and ethanol solutions is illustrated in figure 8-6. Figures 8-6-a and 8-6-b are the representative chromatograms of a processed HPβCD blank sample and HPβCD sample obtained from solubility studies of taxol. These chromatograms showed that there were no interference between chromatograms of taxol and component of solubility, stability and dissolution samples.

8.4 Conclusions

The described assay is rapid and sensitive, allowing for numerous samples to be processed in a short period of time. Perhaps more importantly, this method appears to be a "general" method for the determination of taxol in biological and non-biological samples and in the presence of different solubilizing agents.

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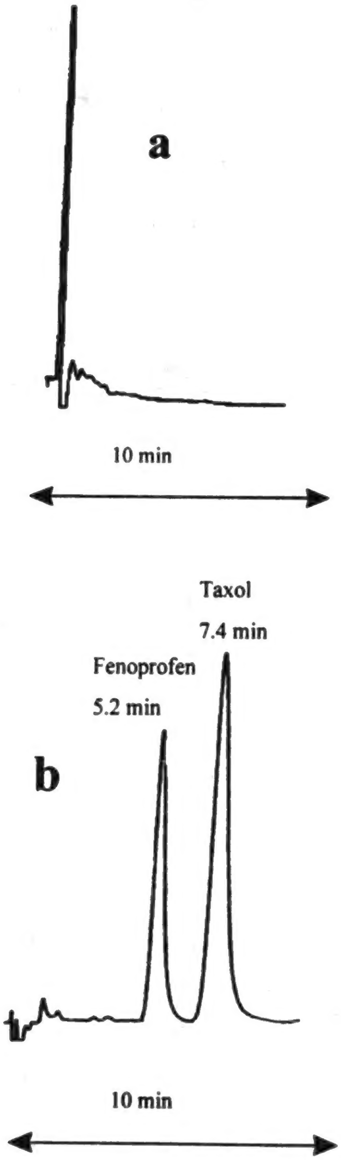

Figure 8-1 Chromatograms of acetonitrile:water (40:60), (a) before and (b) after spiked with taxol (10 μg/ml) and fenoprofen (3.5 μg/ml)

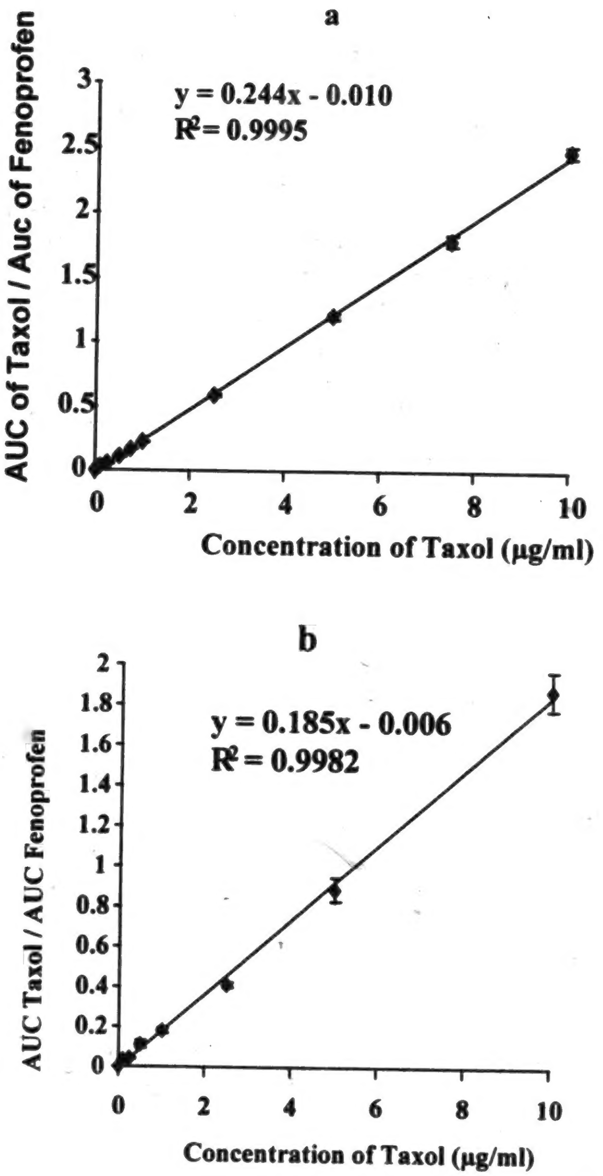

Figure 8-2 Standard Curves of taxol after (a) direct injection of mobile phase spiked with taxol or (b) extract of rats plasma spiked with taxol.

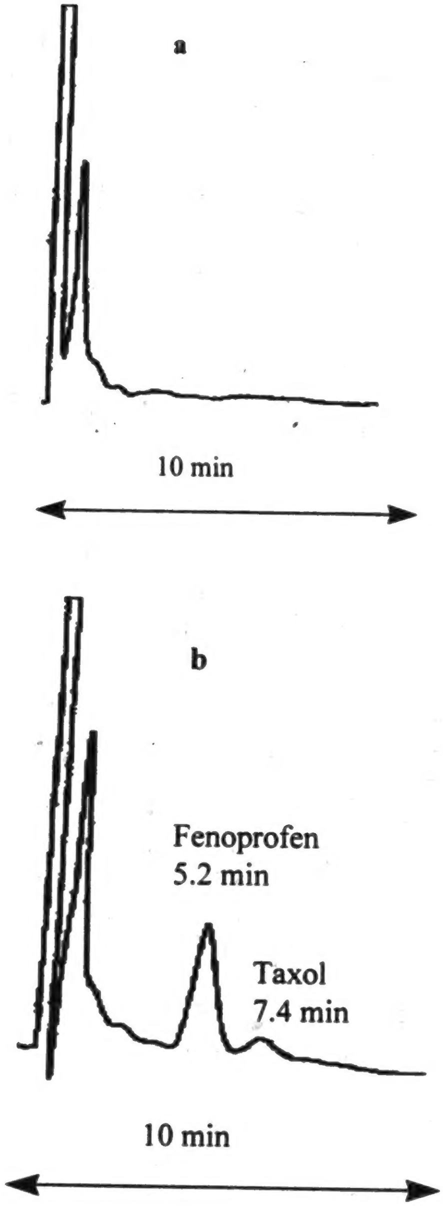

Figure 8-3 Chromatograms of extracts of rat plasma (a) prior and (b) after spiking with taxol (0.1 μg/ml) and fenoprofen (3.5 μg/ml).

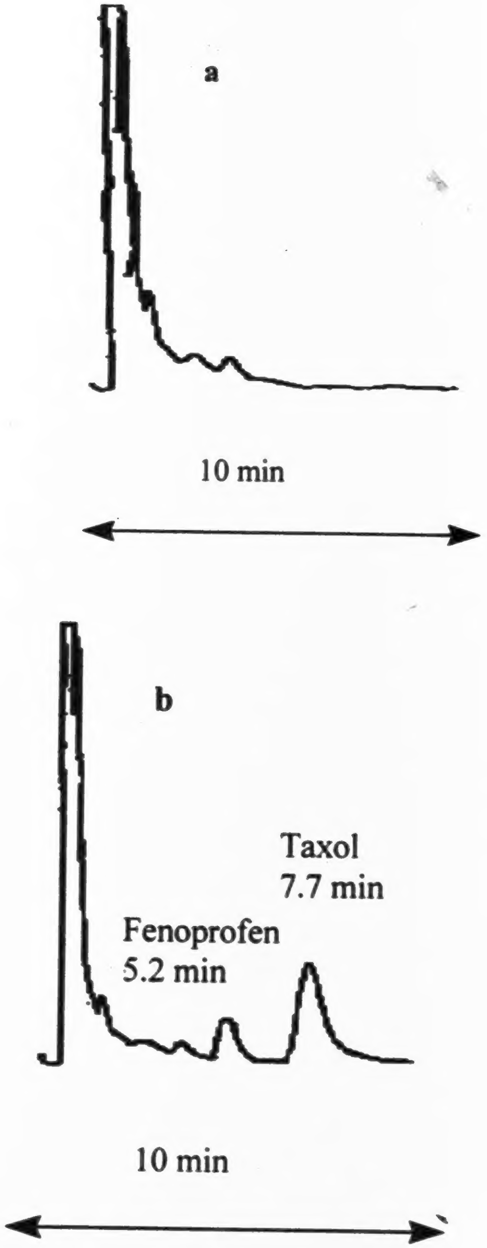

Figure 8-4 **Chromatograms of extracts of rat's blood (a) prior and (b) after spiking with taxol (10 μg/ml) and fenoprofen (3.5 μg/ml).**

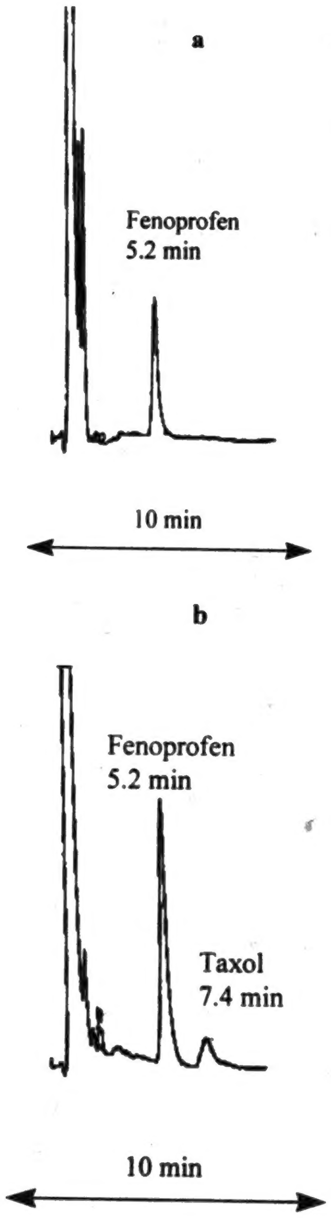

Figure 8-5 **Chromatograms of extracts of plasma from rat obtained (a) prior to and (b) 6 hr after ip injection of taxol (33 mg/kg).**

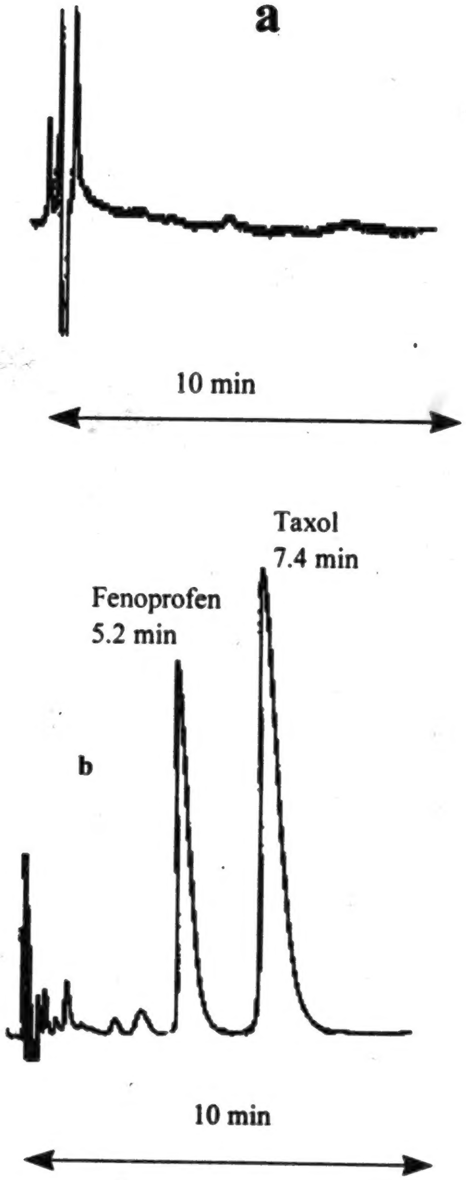

Figure 8-6 **Chromatograms of HPßCD (20% w/v), (a) before and (b) after spiking with taxol (10 μg/ml) and fenoprofen (3.5 μg/ml).**

Chapter 9

9. Mechanisms Involved in Enhancement of In Situ Permeability of Taxol by Nicotinamide and Hydroxypropyl-β-Cyclodextrin: using Immobilized Artificial Membrane (IAM) Chromatography

9.1 Introduction

It is well-known that the major absorption barrier to drugs given orally is the gastrointestinal cell membranes. Most orally administered drugs are absorbed across the intestinal mucosa by a passive diffusion mechanism (Pidgeon *et al.* 1995a). The cell membrane includes the main biological barrier for drug diffusion that results in the absorption of most commercially available drugs. Our finding revealed that the absorption of taxol through rat everted intestine wall is low and not region-selective and an improvement in the absorption of taxol can be achieved by hydroxypropyl-β-cyclodextrins (HPβCD) and nicotinamide. HPβCD and nicotinamide have been reported to improve membrane absorptivity of many drugs by changing the biological condition or physiological condition of cell membrane and/or the physicochemical characteristics of compounds (Hozbor *et al.* 1994, Schipper *et al.* 1993, Matsubara *et al.* 1995, Sugiyama *et al.* 1989, Chaplin *et al.* 1991, Lee *et al.* 1992, Ono *et al.* 1993). We have reported that HPβCD and nicotinamide can improve rats everted intestine permeability characteristics of taxol. Our laboratory has shown that HPβCD and nicotinamide can change the physicochemical characteristics of taxol (solubility, stability, and dissolution), therefore, further studies were undertaken to determine if changes in the physicochemical characteristics of taxol is the predominate factor involved in the permeability enhancement of taxol through rat everted intestine wall.

Permeability coefficient, P_m, of a drug through membranes is directly proportional to partitioning of a drug into fluid membrane, K_m:

$$P_m = (D_m \times K_m) / L$$

where D_m is the membrane diffusion coefficient of the drug and L is the membrane thickness (Pidgeon *et al.* 1995b).

Drug permeability is very difficult to measure, that is why methods to estimate P_m based on the measurements of K_m and D_m have been developed. Measurement of K_m is a key factor to estimate the permeability of compounds. However, K_m is also difficult to be determined *in vivo*. Therefore, some *in vitro* membrane systems [(including Octanol/water partitioning (Pidgeon *et al.* 1995), liposome partitioning (Betageri *et al.* 1989, Betageri and Rogers 1989, Choi and Rogers 1990 and 1991), Chromatographic techniques such as Octadecyl (ODS) reversed phase chromatography (Ong *et al.* 1994) and immobilized artificial membrane (IAM) chromatography (Liu *et al.* 1995, Pidgeon *et al.*, 1990, 1991, 1995a & b, 1996)] have been developed to model drug partitioning into cell membranes.

Although the liposome system represents a more selective model membrane system than a octanol/buffer or ODS chromatography partitioning systems for predicting the membrane permeability and biological activities of many drugs, IAM chromatography seems to be a simpler and faster method (Pidgeon *et al.* 1995a) . Therefore, in order to find out the possible mechanism involved in improving in situ permeability of taxol, the partitioning of taxol through immobilized artificial membrane chromatography columns in which the chromatographic surface emulates the lipid environments was studied. IAM chromatography columns mimic the lipid environment found in gastrointestinal cell membranes. Phosphatidylcholine (PC) is the major phospholipid found in cell membranes and IAM column has been prepared from PC analogues which are models of cell membranes. The possibility of hydrophobic-hydrophilic contribution of drug's partitioning can also be determined by IAM column because those compound which do not have drug-membrane interaction will be eluated from the column faster. It has also been shown that there is a good correlation between those data which has been generated by IAM and those of liposome partitioning, cell culture, and animal models (Pidgeon *et al.* 1995a).

9.2 Materials and Methods

9.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Co, (U.S.A), and used as supplied. Cremophor and Hydroxypropyl-ß-cyclodextrin (molecular weight=1380) were obtained from Aldrich Chemical company (Milwaukee, WI, U.S.A), and were used without further purification. Nicotinamide was supplied from Sigma chemical company (St. Louis, MO, U.S.A). Dulbecco's phosphate buffered saline was purchased from Bio-Whittaker Inc. (Walkorsvillo MD, U.S.A). Rgis IAM-PC-DS HPLC column (Rexchrom) was obtained from Rgis, (Rgis, Morton Grove, Illinois, USA). All other materials were reagent grade. Water was deionized and passed through a Milli-Q apparatus (Millipore) .

9.2.2 IAM Chromatography

In this study, the injection volume was 15 µl of citric acid solution in DPBS or taxol solutions in acetonitrile:water 50:50, cremophor EL, 20% w/v HPβCD, or 20% w/v nicotinamide. Mobile phase was DPBS:acetonitrile (78:22), the flow rate was 1 ml/min, and taxol detection was at 227 nm. Chromatograms were obtained using a Rgis IAM-PC-DS HPLC column (Rgis, Morton Grove, Illinois, USA) and a Waters HPLC System equipped with Waters 600 controller, Waters 996 photodiode array detector (PDA), Waters 717 plus autosampler, and interfered with a NEC computer. Millennium software (version 2.1) was used to control the HPLC system and to record and develop the chromatograms on the computer.

The retention times (T_t) of taxol molecule on IAM chromatography columns were used to calculate taxol capacity factors in different media (k'_{IAM}) using the following equation:

$$k'_{IAM} = (t_t - t_0)/t_t$$

where t_t was the retention time of taxol and t_0 correspond to the column dead time or void volume, which was determined by injection of sodium citrate as non-retained compound.

Taxol required acetonitrile for elution, therefore, the capacity factors from several isocratic elutions at different acetonitrile concentrations were used. Linear extrapolation of plots of log (capacity factor versus log (%acetonitrile) gave the capacity factor at 0% acetonitrile.

9.3 Results and Discussion

Taxol is a lipophilic compound which is retained in an IAM column. In order to facilitate the elution of taxol from IAM column acetonitrile was added to the mobile phase (PBS). Figure 9-1 shows the typical chromatogram of taxol using an IAM column and cremophor formulation. Increasing concentration of acetonitrile in mobile phase decreases the retention time of taxol in the IAM column. Figure 9-2 shows that there is an excellent relationship between log of capacity factor and log %acetonitrile in IAM column (R= 0.9994). The capacity factor of taxol at 0% acetonitrile was calculated to be 24 hrs. Therefore, it seems that because of high lipophilicity, taxol is well retained in IAM column and interacts with membrane. For many drugs this high interaction is an indication of good absorption but in the case of taxol it showed poor absorptivity.

Although partitioning is the major source of drug permeation and variation in drug absorption, passive permeation of drug through cell membranes also depends on the membrane diffusion coefficient of the drug (D_m). D_m significantly depends on the molecular size and/or molecular shape of the drug. As molecular weight increases D_m dramatically decreases. Most orally administered drugs are smaller than the size of membrane lipids that create the barrier to drug absorption, additionally the majority of drugs exhibit sufficient lipophilicity to allow membrane permeability. In the case of taxol, even though it shows very good lipophilicity, factors such as molecular weight and structure have contributed to poor absorption and, consequently low oral bioavailability of this drug.

Table 9-1 shows the retention time of taxol after injection of 25 μl of taxol solution in acetonitrile, cremophor, HPβCD, or nicotinamide into the IAM column using isocratic mobile phase containing acetonitrile:PBS 22:78. No statistically significant difference between the retention time of taxol in those media has been noted, indicating that cremophor, HPβCD and nicotinamide do not change the partitioning of taxol through an IAM stationary column and, presumably, the cell membrane. Although taxol makes complexes with HPβCD and nicotinamide, it seems that this complexation does not change the partitioning of the drug in a cell membrane. Complexation of the drug with cyclodextrin and nicotinamide usually produces a product which has different polarity and molecular weight compared to the drug alone and therefore, one of the consequences of this complexation could be the change in diffusion and partitioning of the drug through the membrane. But in the case of taxol, it seems that the presence of HPβCD and nicotinamide does not change the permeability which is likely due to instability of the complex inside the column. The complexation of taxol with HPβCD and nicotinamide has been shown to be concentration-dependent (Chapter 2). Therefore, because of the dilution of complexed taxol by mobile phase, taxol was released from the cyclodextrin complex with each of the complexing agents allowing to interact with the membrane separately.

Figures 9-3 and 9-4 show the effect of concentration of HPβCD and nicotinamide (0-20% w/v) on partitioning of taxol through the IAM column. No significant changes in retention time of taxol were observed. Therefore, it is concluded that presence of HPβCD and nicotinamide in the range applied does not change the partitioning of taxol in a cell membrane. Furthermore the presence of different concentrations of these complexing agents did not overcome the diluting effect of the mobile phase.

9.4 Conclusions

It is concluded that the permeability enhancement effect of HPβCD and nicotinamide in the everted rat intestine experiment may be due to change in the permeability behavior of the intestinal cell membrane or oral availability of taxol rather than change in partitioning of taxol in the cell membrane.

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Table 9-1 Retention time and capacity factor of taxol in IAM column in the presence of different solubilizing agents (acetonitrile, cremophor, HPβCD, or nicotinamide) using and isocratic mobile phase containing acetonitrile:PBS 22:78 and injection of 25 μl of taxol's solution.

	Retention time (t)	Capacity factor (k_{IAM})
Taxol-ACN/H2O	9.13 ± 0.03	5.51 ± 0.008
Taxol-Cremophor EL	9.11 ± 0.01	5.50 ± 0.039
Taxol-HPBCD	9.09 ± 0.05	5.42 ± 0.38
Taxol-Nicotinamide	8.99 ± 0.05	5.52 ± 0.023

Results are reported as means ± SEM.

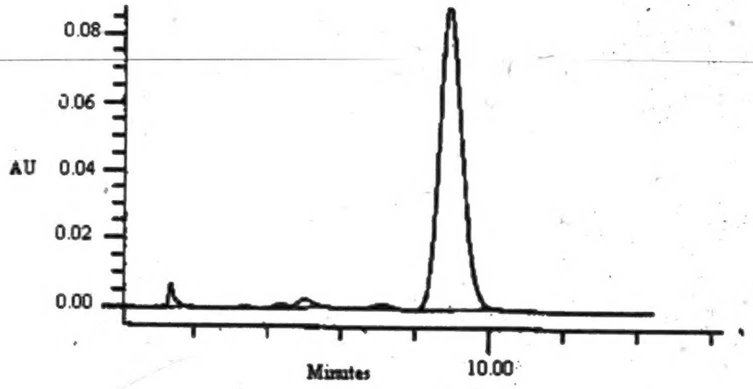

Figure 9-1 Chromatogram of taxol using IAM column and an isocratic mobile phase containing acetonitrile:PBS 22:78.

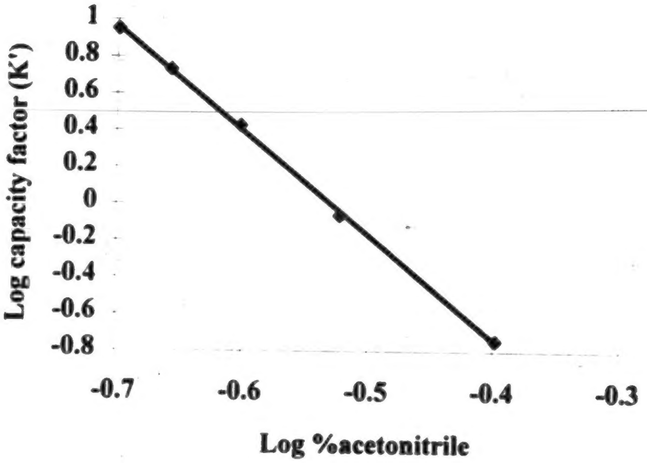

Figure 9-2 **Correlation between log capacity factor of taxol in IAM column and log concentration of acetonitrile in the mobile phase.**

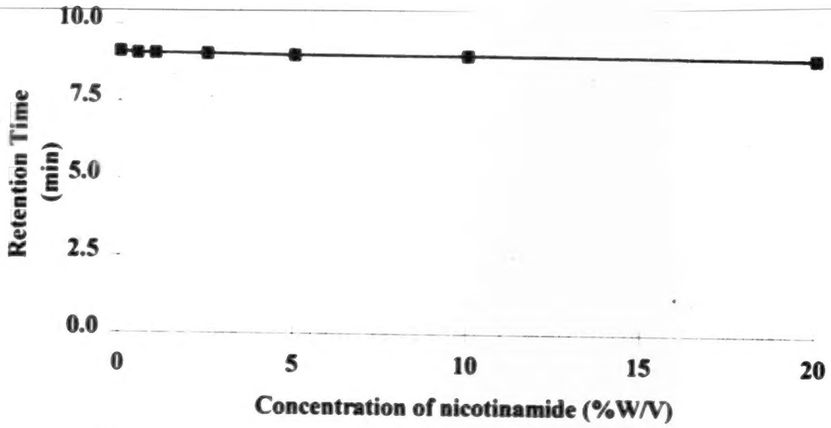

Figure 9-3 **Effect of nicotinamide concentration on retention time of taxol in IAM column**

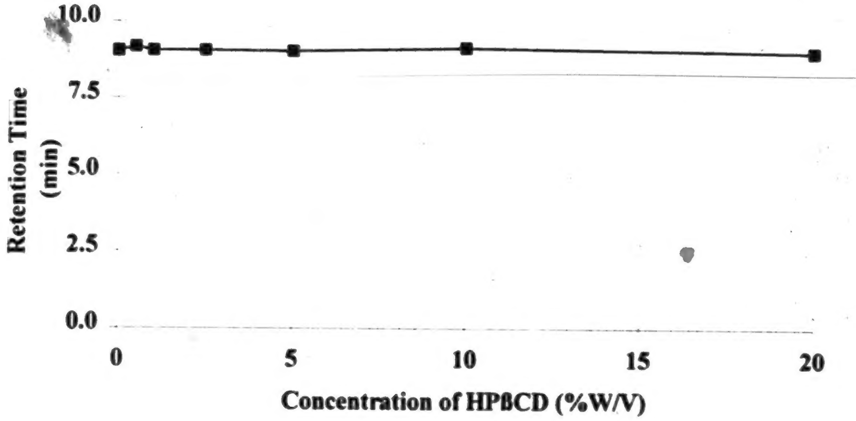

Figure 9-4 Effect of HPβCD concentration on retention time of taxol in IAM column

Chapter 10

10. Improving Oral Bioavailability of Taxol

10.1 Introduction

Clinical use of taxol has often been limited by its side effects which include hypersensitivity reactions due to the rate and route of administration (iv) of its cremophor formulation (Koeller and Dorr, 1994). It has been reported that this kind of hypersensitivity reaction is associated with all drugs which have cremophor in their formulations. Changing the route of administration and elimination of cremophor from their formulations decreased or eliminated the incident of hypersensitivity reactions (Howric *et al.* 1985 and Koeller and Dorr, 1994). We intended to examine the oral route as an alternative to the iv route for administration of taxol. The oral route is the most convenient and the most widely used in the drug delivery. During the process of drug development, oral bioavailability of a compound is a critical factor for the future of that compound as a drug. Different figures have been reported for oral bioavailability of taxol but these data reveal the poor oral absorption of taxol. Oral bioavailability is primarily controlled by the process of drug solubility, dissolution, stability and absorption across the intestinal epithelium. Our previous *in vitro* studies have shown that taxol is a poorly soluble compound and its dissolution rate is limited by its solubility. Also, we have found that although taxol has enough lipophilicity to pass through the gut membrane, its permeation is limited by the lack of diffusivity due to its large molecular size. We have shown that presence of hydroxypropyl-β-cyclodextrin (HPβCD) and nicotinamide increase solubility, dissolution rate, stability, and permeability of taxol by complexation between taxol and these compounds and changing the biological condition of the gut wall. In this study we intended to investigate the possibility of increasing oral bioavailability of taxol by administering taxol in HPβCD or nicotinamide formulations.

10.2 Materials and Methods

10.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA, and used as supplied. $[H^3]$Taxol was obtained from NCI (USA) as a gift. Cremophor and hydroxypropyl-ß-cyclodextrin (molecular weight=1380) were obtained from Aldrich Chemical Company, Milwaukee, WI, USA, and were used without further purification. Nicotinamide was supplied by Sigma Chemical Company, St. Louis, MO, USA. Methoxyfluran (Metofan) was supplied by Pitman-Moore Ltd. (Mississauga, Canada). Heparin (Hepalean) was supplied by Organon Teknika, (Toronto, Canada). Disposable syringes (1 ml, 5 ml), needles (23 G), PE-10 and PE-50 tubing were obtained from Becton and Dickinson (Company, Rutherford, NJ, USA). Water was deionized, Milli-Q (Millipore) purified. All other materials were reagent grade.

10.2.2 Bioavailability Studies

10.2.3 Surgical Methods and Sample Collection

The day before drug administration, silastic catheters (0.025" i.d. X 0.047" o.d.; Dow-Corning, Midland MI) were implanted into the right jugular vein of male Sprague Dawley rats and the animals were allowed to recover overnight. All surgical procedures were performed under anesthesia, using diethyl ether for induction and methoxyfluran for maintenance. Rats were fasted for 12 hr prior to and 2 hr after drug administration. All animals were housed in plastic metabolic cages during experiments, with free access to water. Drug doses were given intraperitoneally by needles (23 G) orally by gastric intubation or intravenously as bolus doses into the jugular vein cannula. Following iv doses, the cannulas were flushed with 0.5 ml saline solution. Serial blood samples (250 ml) were then collected through the jugular vein cannula. After each sample the catheters were flushed with an equal volume of heparin in saline (100 units/ml). Each blood sample was immediately centrifuged and 100 µl plasma was separated and stored at -20° C until assayed for taxol.

10.2.4 Drug Administration

Intravenous (10 mg/kg, n=3) and ip (40 mg/kg, n=3) doses of taxol in cremophor formulation (10 mg/ml) or oral (40 mg/kg, n=3) doses of taxol in cremophor, HPβCD, or nicotinamide formulation (10 mg/ml) were administered to unanesthetized jugular vein cannulated Sprague Dawley rats. Blood samples (250 μl) were collected at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hrs after dosing. After iv dose an additional blood sample at 0.25 hr was collected from each animal.

10.2.5 Drug Assay

10.2.5.1 Chromatography

A Waters HPLC (Waters Lc module 1) consisting a Model 600E pump, a 715 auto-injector, and a 486 multi-wavelength UV detector (Waters, Mississauga, Canada) was operated at ambient temperature. The column was a partisil ODS (phenomenex ® 100 mm, 4.6 mm ID, 5 μm particle size) protected by a partisil ODS (phenomenex ® 30 mm, 4.6 mm ID, 5 μm particle size) guard column. The mobile phase consisted of 40% acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.0) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm.

10.2.5.2 Extraction Procedure

Plasma samples were analyzed for taxol as described before (chapter 8). In brief, 0.1 ml of internal standard (fenoprofen) and 1 ml ACN were added to 0.1 ml of plasma sample, and the mixtures were vortexed for 1 min and centrifuged at 1800 g for 5 min. The supernatant was transferred to clean tubes and evaporated to dryness. The residues were reconstituted to 0.2 ml with ACN: H_2O (50:50) and 50 μl injected into the HPLC system. For standard curve and calculation of extraction yield, drug free rat plasma was spiked with solutions of taxol to give taxol concentration of 0, 0.05, 0.1, 0.25, 0.50, 1, 2.5, 5, 10, and 25 μg/ml. After these samples were extracted and chromatographed, the peak areas of taxol were compared with identical concentrations of the drug in ACN:H_2O (50:50) which were directly injected onto the HPLC. Percent recovery was calculated as the peak area of extracted drug divided by the peak area of the unextracted drug. The limit of detection was taken as a signal-to-noise ratio of 4:1. Identification of peaks corresponding to the taxol was accomplished by chromatography of the drug.

10.2.5.3 Liquid Scintillation Spectrometry

A Beckman LS 9000 (Beckman Co., Fullerton, CA, USA) Liquid Scintillation Spectrometer was employed. Samples (plasma or urine) were transferred to a scintillation vial and 10 ml of scintillation cocktail (Fisher ready safe liquid scintillation cocktail) was added to that, and the mixture was vortex-mixed for 1 min before being put into the LS-9000 scintillation counter for determination of radioactive content.

10.2.6 Data Analysis

10.2.6.1 Pharmacokinetic Indices

The terminal elimination half-life ($t_{1/2}$) for taxol was determined by 0.693/β, where β (elimination rate constant) was calculated using the regression slope of the log-linear terminal elimination phase. The area under plasma concentration versus time curve ($AUC_{0-\infty}$) for taxol was determined using linear trapezoidal rule for 0 hrs to the time of last concentration (C_{last}). The extrapolated AUC ($AUC_{t-\infty}$) was calculated as C_{last}/β. The $AUC_{0-\infty}$ (AUC) was calculated by adding AUC_{0-t}+ $AUC_{t-\infty}$. Systemic clearance was calculated by dividing the administered iv dose by the corresponding $AUC_{0-\infty}$. The volume of distribution was calculated by $V_d = Cl_{sys}/\beta$. The oral clearance (CL_{Oral}) was determined by $Cl_{oral} = F \times dose_{po}/AUC_{0-\infty, po}$. Absolute bioavailability (F) was calculated by dividing the mean AUC_{po} or AUC_{ip} by the mean AUC_{iv} after the same dose.

10.3 Results and Discussion

10.3.1 Iv Pharmacokinetic Parameters

Figure 10-1 presents the time course of plasma concentration of taxol in rats after iv, ip, and oral administration. Each point in the figure represents the mean of 3 rats. The plasma concentration of taxol declined rapidly after injection. This was followed by a more prolonged terminal phase. It seems that disappearance of taxol from plasma is best characterized by a biexponential elimination model. Calculation of pharmacokinetic parameters of taxol based on noncompartmental model showed that the terminal elimination rate constant and elimination half-live of taxol after iv administration of 10 mg/kg dose of the drug are 0.08 hr^{-1} and 9.09 hrs, respectively.

The calculated volume of distribution was 1162 ml. This value is much larger than the volume of rat total body water, indicating that taxol is extensively bound to proteins and/or other tissue elements, possibly tubulin and other tissue proteins. Plasma protein binding, as has been determined by both equilibrium dialysis and ultrafiltration methods, has been reported to range from 95% to greater than 97% over a wide range of drug concentrations (Longnecker *et al.*, 1986; Wiernik *et.al.*, 1987a,b). However, despite extensive protein binding to plasma proteins, taxol is readily eliminated from the plasma compartment, suggesting reversible binding of lower affinity. The systemic clearance value was determined to be 90 ml/hr/kg.

10.3.2 Intraperitoneal Bioavailability

After intrapritoneal (ip) administration to male rat, (Figure 10-1), taxol clearance (CL_{tb}) was 1248 ml hr^{-1} kg^{-1} and the terminal half-life ($t_{1/2}$) was 22.14 hrs. The terminal elimination rate constant and the bioavailability of taxol was approximately 0.03 hr^{-1} and 7.1%, after ip, administration, respectively. Large amount of taxol was detected in the peritoneal cavity of rat, 24 hrs after ip delivery of the drug indicating uncompleted absorption of taxol after ip administration. Other pharmacokinetic parameters such as half-life and volume of distribution were much higher than that associated with iv bolus injection (Table 10-1). Maximal plasma concentrations (C_{max} = 0.98 μg/ml) were achieved within 2 h of ip administration. At a dose of 33 mg/kg, the plasma drug concentration was higher than the *in vitro* cytotoxic concentration range of taxol (0.1-10 μmol/L).

10.3.3 Oral Bioavailability

Taxol was not detected in the plasma of rats after oral delivery of the drug as suspension (taxol in water, 30 mg/kg) or solution (cremophor formulation, 10 mg/kg). After oral administration of tritiated taxol in cremophor formulation (10 mg/kg taxol plus 10 μCi tritiated taxol/kg) tritiated taxol was detected in plasma and urine. Since no metabolite of taxol has been detected in plasma and urine in applied doses, (Rizzo *et al.*, 1990), we assumed that all detected level of radioactivity is because of taxol and not a metabolite of it. After 24 hrs 0.01% of total tritiated taxol was detected in urine. Taxol was detected in the plasma of 1 out of 3 rats which were treated with 40 mg/kg of soluble taxol in cremophor formulation (Figure 10-1). Based on those data which were collected from that rat, the oral bioavailability of taxol was calculated to be 0.16% which is very low.

10.3.4 Effect of HPßCD and Nicotinamide on Bioavailability of Taxol

Figures 10-2 and 10-3 show the plasma concentration of taxol in rats after oral administration of taxol (40 mg/kg) in HPßCD and nicotinamide formulations. Calculation of pharmacokinetic parameters of taxol based on noncompartmental model (Table 10-1) showed that HPßCD and nicotinamide increased the oral bioavailability of taxol by 6 and 14.6 folds respectively. The C_{max} and the terminal elimination half-life, of taxol after administration in HPßCD are 0.17 (μg/ml) and 16.29 hrs and in nicotinamide formulations are 0.67 (μg/ml) and 14.50 hrs, respectively. We have already shown that HPßCD and nicotinamide can increase the solubility, dissolution and permeability of taxol. Therefore, increase in bioavailability of taxol may be explained by increase in dissolution and permeability of taxol in gastrointestinal tract in the presence of HPßCD and nicotinamide.

10.4 Conclusions

HPßCD and nicotinamide can significantly increase the oral bioavailability of taxol achieving the substantial absorption of taxol which can be clinically important. From the results of solubility, dissolution and permeation studies, it appears that HPßCD and nicotinamide facilitate the absorption of taxol mostly by changing the physiological condition of gastrointestinal. Further studies are required to study the exact mechanism of absorption enhancer effects of HPßCD and nicotinamide and GI toxicity of these absorption enhancers.

10.5 References

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Table 10-1 **Noncompartmental pharmacokinetic parameters of taxol in rat. Data are presented as the mean (n=3). For po administration taxol was detected in plasma of only one of three rats.**

	iv	ip	po (Cremophor)	po (HPβCD)	po (Nicotinamide)
Dose (mg/kg)	10	33	40	40	40
C_{max} (μg/ml)	100	0.98	0.37	0.17	0.67
T_{max} (hr)	0	2	2	1	2
AUC (μg.hr/ml)	112.84	26.44	0.73	4.38	10.69
β (hr^{-1})	0.08	0.03	1.50	0.04	0.07
$t_{1/2}$ (hr)	9.09	22.14	0.46	14.17	10.42
CL (ml/hr/kg)	89	87.61	87.6	88.58	86.50
V_d (ml/Kg)	1162	2954	58.4	2215	1236
F	100	7.1	0.16	0.97	2.37

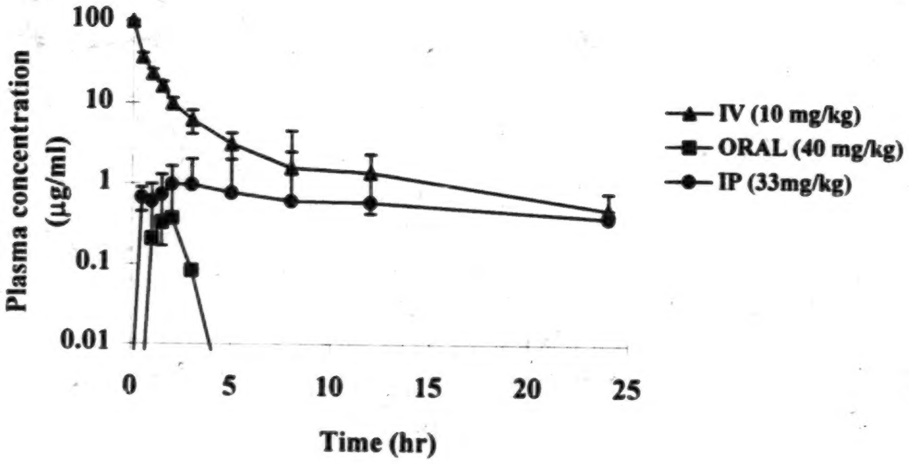

Figure 10-1 Plasma concentration-time profile of taxol in rat after iv, ip, and oral administration of taxol. For ip and iv administrations data are presented as the mean ±SEM (n=3). For po administration taxol was detected in plasma of only one of three rats.

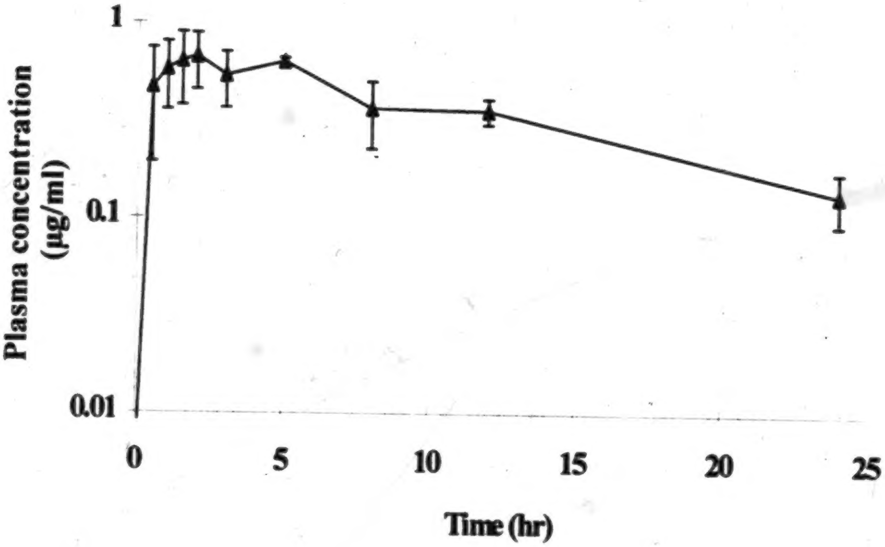

Figure 10-2 Plasma concentration-time profile of taxol in rat after oral administration of taxol in nicotinamide formulation. Data are presented as the mean ±SEM (n=3).

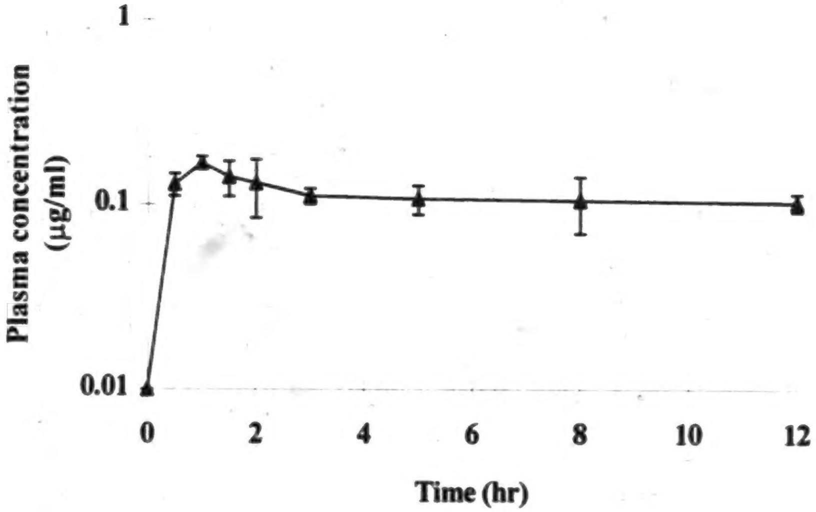

Figure 10-3 Plasma concentration-time profile of taxol in rat after oral administration of taxol in HPβCD formulation. Data are presented as the mean ±SEM (n=3).

Chapter 11

11. Intestinal Permeability and Bioavailability of Taxol in the Presence of HPßCD, Nicotinamide, and NSAIDs

11.1 Introduction

Taxol is a remarkable anticancer agent with poor oral absorption. Theoretically the absorption of compounds across mucosal gut membranes may occur via one of these series of processes: simple diffusion, perorption, specific transport mechanisms including facilitated diffusion, exchange diffusion, active transport, and pinocytosis.

Our previous results primarily support the possibility of passive permeation of taxol through gut wall which was very slow and poor. Also, we have shown that presence of HPßCD and nicotinamide on the site of absorption does not significantly change the partitioning of taxol through phospholipid layer of mucosal membrane. Therefore, it has been concluded that the permeation enhancer effect of HPßCD and nicotinamide may be due to the effect of HPßCD and nicotinamide on the biological condition of cell membrane. Intestinal permeation is a complex process reflecting at least 3 particular unmediated permeation pathways of transmucosal diffusion including: the intercellular junction between adjacent entrocyts (tight junctions), aqueous transmembrane pores in the enterocyte brush border membrane, and the lipid rich hydrophobic pathway in the brush border. Taxol is a bulky structural compound with an octanol/phosphate buffer partitioning of more than 99. Therefore, it seems that the most dominant permeation pathway of transmucosal diffusion of taxol could be through the tight junctions of the gut wall.

Different analytical techniques have been assessed for *in vivo* intestinal permeability. The most commonly used markers are the urinary excretion following oral ingestion of carbohydrates, (i.e. lactose, cellobiose, and manitol), ethylene glycol polymers such as polyethylene glycol (PEG), and non-degradable radionuclide probes such as ^{51}Cr-EDTA (Abbakken *et al.* 1989, Juby *et al.* 1989, Tagesson *et al.* 1984)

All of the current intestinal permeability marker have advantages and disadvantages and none passes all of the criteria of an ideal markers (Cooper *et al.* 1984). However, because of the water solubility, stability and γ-ray emission, ^{51}Cr-EDTA technique seems to have more analytical advantages over other methods. In addition ^{51}Cr-

EDTA is stable and has a half-life of one month so that aliquots of radioactive material can be easily stored.

In the current study the *in vivo* intestinal permeability of taxol in the presence of HPßCD and nicotinamide was assessed by ^{51}Cr-EDTA techniques to delineate the mechanism of permeation enhancer effects of HPßCD and nicotinamide. It has been shown that NSAIDs may change the function of tight junctions by changing the endogenous prostaglandin levels (Davis 1996), therefore, we studied the effect of NSAIDs (flurobipropfen) on the permeation of taxol to assess the mechanism of permeation enhancer effects of HPßCD and nicotinamide and potential application of NSAIDs as an absorption enhancer for taxol.

11.2 Material and Methods

11.2.1 Materials

Taxol was purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA, and used as supplied. Cremophor and hydroxypropyl-ß-cyclodextrin (molecular weight=1380) were obtained from Aldrich Chemical Company, Milwaukee, WI, USA, and were used without further purification. Nicotinamide and flurbiprofen were supplied by Sigma Chemical Company, St. Louis, MO, USA. ^{51}Cr-EDTA (specific activity 570 MC/mg)was purchased from Dupont NEN (Wilmington, DE, USA). Methoxyfluran (Metofan) was supplied by Pitman-Moore Ltd. (Mississauga, Canada). Heparin (Hepalean) was supplied by Organon Teknika, (Toronto, Canada). Disposable syringes (1 ml, 5 ml), needles (23 G), PE-10 and PE-50 tubing were obtained from Becton and Dickinson (Company, Rutherford, NJ, USA). Water was deionized, Milli-Q (Millipore) purified. All other materials were reagent grade.

11.2.2 Bioavailability Studies

11.2.2.1 Surgical Methods and Sample Collection

The day before the drug administration, silastic catheters (0.025" i.d. X 0.047" o.d.; Dow-Corning, Midland MI) were implanted into the right jugular vein of male Sprague Dawley rats and the animals were allowed to recover overnight. All surgical procedures were performed under anesthesia, using diethyl ether for induction and methoxyfluran for maintenance. Rats were fasted for 12 hrs prior to and 2 hr after drug administration. All animals were housed in plastic metabolic cages during experiments, with free access to water. Drug doses were given orally by gastric intubation. Serial blood samples (250 µl) were then collected through the jugular vein cannula. After each sample, the catheters were flushed with an equal volume of heparin in saline (100 units/ml). Each blood sample was immediately centrifuged and 100 µl plasma was separated and stored at -20° C until assayed for taxol.

11.2.2.2 Drug Administration

Intravenous (10 mg/kg, n=3), Oral (40 mg/kg n=3), doses of taxol in cremophor, cremophor+20% w/v HPβCD, or cremophor+20% w/v nicotinamide formulations (10 mg/ml) were administered to Sprague Dawley rats. Blood samples (250 µl) were collected from jugular vein at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hr after dosing. To examine the effect of NSAIDs, flurbiprofen (0, 1, 5, 10 mg/kg) was suspended in 0.5 ml of 1% methylcellose and administered orally to each group of rats (n=3) half-hr before administration of taxol in cremophor formulation (40 mg/kg, 10 mg/ml).

11.2.2.3 Drug Assay

A Waters HPLC (Waters Lc module 1) consisting a Model 600E pump, a 715 auto-injector, and a 486 multi-wavelength UV detector (Waters, Mississauga, Canada) was operated at ambient temperature. The column was a partisil ODS (phenomenex ® 300 mm, 4.6 mm ID, 5 µm particle size) protected by a partisil ODS (phenomenex ® 30 mm, 4.6 mm ID, 5 µm particle size) guard column. The mobile phase consisted of 40%

acetonitrile in water containing 0.05% v/v glacial acetic acid (pH 4.0) at a flow rate of 1.7 ml/min. The eluate was monitored at 227 nm.

11.2.2.4 Pharmacokinetic Indices

The terminal elimination half-life ($t_{1/2}$) for taxol was determined by 0.693/β, where β (elimination rate constant) was calculated using the regression slope of the log-linear terminal elimination phase. The area under plasma concentration versus time curve ($AUC_{0-\infty}$) for taxol was determined using linear trapezoidal rule for 0 hrs to the time of last concentration (C_{last}). The extrapolated AUC ($AUC_{t-\infty}$) was calculated as C_{last}/β. The $AUC_{0-\infty}$ was calculated by adding AUC_{0-t}+ $AUC_{t-\infty}$. Systemic clearance was calculated by dividing the administered iv dose by the corresponding $AUC_{0-\infty}$. The volume of distribution was calculated by $V_d = Cl_{sys}/\beta$. The oral clearance (CL_{Oral}) was determined by $Cl_{oral} = F \times dose_{po}/AUC_{0-\infty,\ po}$. Absolute bioavailability (F) was calculated by dividing the mean AUC_{po} or AUC_{ip} by the mean AUC_{iv} after the same dose.

11.2.3 Intestinal Permeability Experimentation

To measure intestinal permeability, 0.5 ml of a solution containing 10 µci/ml of ^{51}Cr-EDTA was administered orally following the last sampling of taxol bioavailability studies. An 18 gauge 4 cm curved feeding needle attached to a 1 ml syringe was used to deliver solution. Following the administration of the ^{51}Cr-EDTA solution urine was collected from 0 to 8 hrs. The urine was collected in cups and after each collection period 10 ml of distilled water was used to rinse the urine collection trays and was transferred to scintillation vials.

11.2.3.1 Measurement of Radioactivity in the Urine Samples After Oral Administration of ^{51}Cr-EDTA

Urine samples were counted directly in a Beckman Gamma 8000 multisample counter (Beckman, Irvine, CA) for 1 minute in a counting window scanning within a range of 0-2 Mev. Two blank samples (10 ml tap water) and two standard samples containing 10 ml blank urine plus 100 µl of the administered ^{51}Cr-EDTA solution were counted with every series of urine samples. Relative permeability was determined by calculating the activity present in each urine sample as a percent of the administered dose after correcting for background radiation. The apparent permeability is reported as the percent of excretion in urine from 0 to 8 hr.

11.2.4 Statistical Evaluation

Statistical evaluations were performed on all data using One-way ANOVA. Where a significant F-value was obtained, the Student-Newman-Keuls method was used for pairwise multiple comparisons, the level of significance chosen for all statistical analyses being $\alpha = 0.05$. The results were expressed as mean ± standard error of the mean (SEM).

11.3 Results and Discussion

11.3.1 HPßCD and Nicotinamide

HPßCD and nicotinamide significantly increased the oral bioavailability of taxol. The administration of taxol in formulations of cremophor in 20% w/v HPßCD or in 20% w/v nicotinamide increased the oral bioavailability of taxol 6- and 14.6-fold, respectively, compared to cremophor alone (Table 11-1). Previously it was shown that HPβCD and nicotinamide increased the solubility, dissolution, and stability of taxol, and that the rate limiting step in the process of oral absorption of taxol is its low permeability. Although addition of HPßCD and nicotinamide significantly enhanced the permeability of taxol in the everted rat intestine, results of partitioning of taxol in the IAM column chromatography suggested that this did not play a significant role. Instead changes in the biological condition of cell membrane at the tight junctions is thought to be important factor. ^{51}Cr-EDTA is a marker which can be absorbed through the tight junctions of gut wall. In normal biological condition the absorption of ^{51}Cr-EDTA is very low, but in the presence of permeation enhancers, GI toxic drugs (i.e., NSAIDs, corticosteroids, ethanol, progesterone, methotrexate, ergotamine), and some disease such as (Cystic fibrosis, coliac disease, Chronic alcoholism) the absorption of ^{51}Cr-EDTA is increased and the percentage excreted in urine raises up to 40% (in case of indomethacin) in the first 8 hr after administration of this marker (Davies 1996). In order to assess the GI toxicity of HPßCD and nicotinamide, after 24 hrs of oral administration of taxol, (cremophor, cremophor + HPßCD or cremophor + nicotinamide formulations), ^{51}Cr-EDTA solution was administered and the %^{51}Cr-EDTA excreted in urine was measured. Figure 11-1 compares the ^{51}Cr-EDTA excreted in urine in the first 8 hr after administration of ^{51}Cr-EDTA. It shows that although there is a significant difference between intestinal permeability enhancing effect of cremophor, HPßCD and nicotinamide, the GI toxicity of HPßCD and nicotinamide is not severe or at least it is reversible after 24 hr. These data may support the idea that HPßCD and nicotinamide change the bioavailability of taxol through changes in the biological condition of the intestine and most likely through changes in the function of tight junctions of the cell membrane.

11.3.2 NSAIDs

The therapeutic effects and major toxic side effects of NSAIDs have been attributed to the ability of these drugs to inhibit the synthesis of stable prostaglandins (PGs), through the direct inhibition of prostaglandin H syntheses. The most apparent side-effects of NSAID use in the lower intestine are manifested as increased intestinal permeability, bleeding, diaphragm-like strictures, ulceration, perforation, and in severe cases, hemorrhage and death.

The mediators that regulated the permeability of the tight junction have not been clearly delineated but may be influenced by a number of factors including the cyclic AMP tricarboxylic acid cycle and anaerobic glycolysis, cytoskeletal structure, neutrophils, calcium, nitric oxide, and cytokines (Davies 1996). However, it seems that this is largely due to cAMP-dependent mechanisms. The concentration of cAMP in the epithelial cell, and in turn the permeability of tight junctions, may be influenced by the levels of endogenous prostaglandins. Thus it is reasonable to assume that changes in endogenous prostaglandin levels by NSAIDs may result in changes in the function of tight junctions (Abramson, *et al.* 1989 and Davis 1996).

The plasma time profile of taxol after co-administration of taxol and flurbiprofen is shown in figure 11-2. Flurbiprofen shows a significant effect on bioavailability of taxol. Pretreatment of rats with 10 mg/kg suspension of flurbiprofen in 1% methylcellose 0.5 hr before administration of taxol increased the bioavailability of taxol from 0.16% to 23%. Figure 11-3 illustrate the dose-dependency of flurbiprofen on the bioavailability of taxol. At dose of 1 mg/kg flurbiprofen did not show any effect on bioavailability of taxol, and taxol was only detected at time 2 and 3 hrs in 2 out of 4 treated rats. However, pretreatment of rats with 5 and 10 mg/kg of flurbiprofen 0.5 hrs before the administration of taxol, enhanced the bioavailability of taxol, 48- and 143-fold, respectively (Table 11-1). It has been shown that the intestinal permeation enhancer effect of NSAIDs is not only depend on the chemical structure of NSAIDs but also on dose (Davies 1996). Therefore, dose-dependent enhancement in oral bioavailability of taxol in the presence of flurbiprofen can be explained by the higher dose and greater effect of flurbiprofen in opening the tight junctions of rat intestine and therefore, higher permeation of taxol transport into the systemic circulation and consequently higher bioavailability. These data may support the idea that permeation is the main important parameter in low bioavailability of taxol and it is the rate-limiting step in oral absorption of taxol.

The effect of different doses of flurbiprofen on ^{51}Cr-EDTA excreted in urine in the first 8 hr after administration of % ^{51}Cr-EDTA (24.5 and 24 hrs after administration of flurbiprofen and taxol respectively) is illustrated in figure 11-3. The % ^{51}Cr-EDTA excreted in urine at 1 mg/kg dose of flurbiprofen is about the control and cremophor alone, while after 5 and 10 mg/kg doses of flurbiprofen the % ^{51}Cr-EDTA excreted in urine increased 50 and 90 %, respectively. Coadministration of flurbiprofen and taxol at the same time increased the ^{51}Cr-EDTA excreted in urine from 6.3% (± 0.76 SEM) to 8% (single point study) showing a better permeation enhancement effect.

11.4 Conclusions

These data support the idea that permeation is the rate-limiting step in oral absorption of taxol. The mechanism of permeation enhancer effects of HPßCD and nicotinamide is through the effect of these compound on tight junctions of intestinal wall. Also it was shown that NSAIDs in general, and flurbiprofen, in particular, are potent permeation enhancers for increasing the oral bioavailability of taxol. Coadministration of taxol with flurbiprofen increased the bioavailability of taxol from 0.16% to 23%.

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Table 11-1 **Noncompartmental pharmacokinetic parameters of taxol in rat after administration of taxol in cremophor (CR, control), HPβCD, and Nicotinamide (ND) formulations or administration of taxol in cremophor formulation with pretreatment of rats with 5 and 10 mg/kg of flurbiprofen (FB). Data are presented as the mean (n=3). In control group (no pretreatment) taxol was detected only in plasma of one of three rats.**

	iv	Oral (CR) (Control)	Oral (HPβCD)	Oral (ND)	Oral CR + (FB) (5 mg/kg)	Oral CR + (FB) (10 mg/kg)
Dose (mg/kg)	10	40	40	40	40	40
C_{max} (μg/ml)	100	0.37	0.17	0.67	3.57	11.9
T_{max} (hr)	0	2	1	2	2	8
AUC (μg.hr/ml)	113	0.73	4.38	10.7	35.1	104
β (hr^{-1})	0.08	1.50	0.04	0.07	0.06	0.12
$t_{1/2}$ (hr)	9.09	0.46	14.17	10.42	11.74	6.01
CL (ml/hr/kg)	89	87.61	88.58	86.50	88.59	88.62
V_d (ml/Kg)	1162	58	2215	1236	1477	738.5
F	100	0.16	0.97	2.37	7.77	23.04

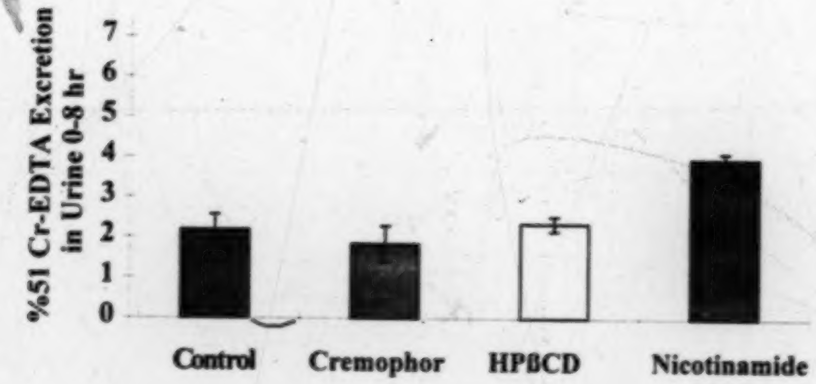

Figure 11-1 Effect of formulation of taxol on ^{51}Cr-EDTA urinary excretion 0-8 hr. Data are presented as the mean ±SEM (n=3). Only nicotinamide formulation is significantly different from others.

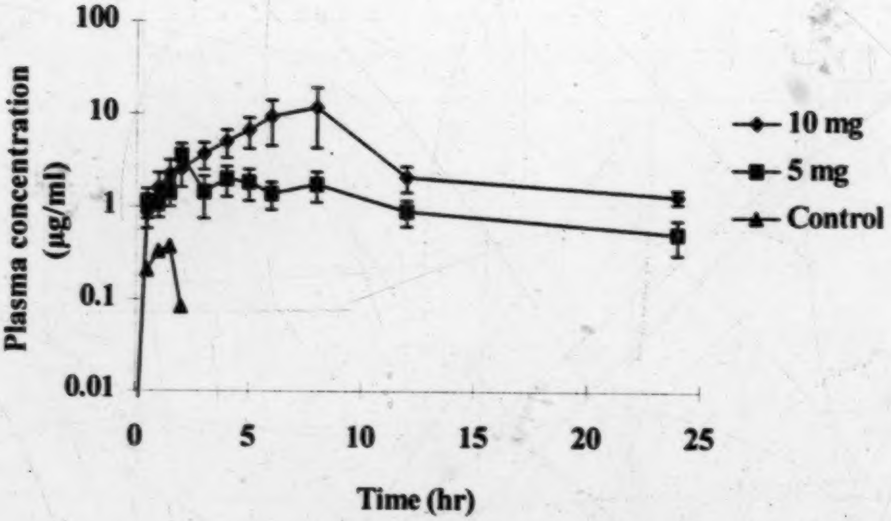

Figure 11-2 Effect of pretreatment of rats with oral formulation of flurbiprofen (1-10 mg/kg) on plasma concentration-time profile of taxol (40 mg/kg). Data are presented as the mean ±SEM (n=3). After pretreatment with 1 mg/kg dose of flurbiprofen, taxol was detected only in 2 hr and 3 hr plasma samples of two of four treated rats. In control animals (no pretreatment) taxol was detected only in plasma of one of three rats.

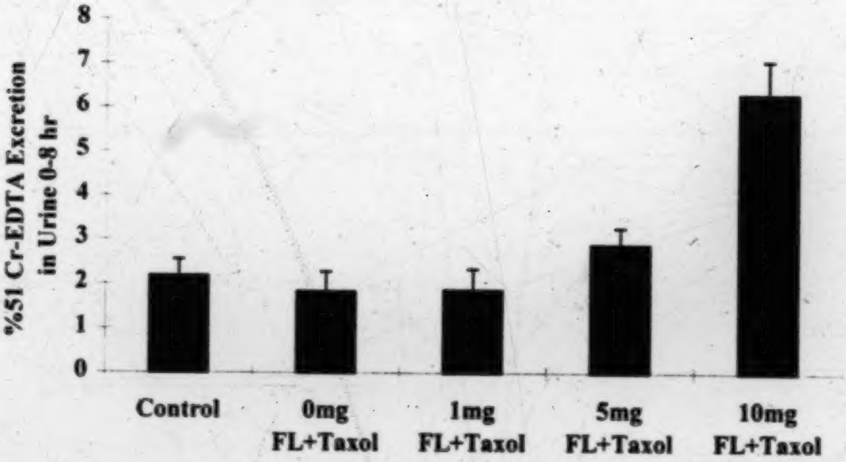

Figure 11-3 Effect of a different dose of flurbiprofen (FL) on ^{51}Cr-EDTA urinary excretion 0-8 hr. Data are presented as the mean ±SEM (n=3). Only 10 mg FL is significantly different from others.

Chapter 12

12. General Discussion and Conclusions

12.1 Introduction

Taxol is one of the more intriguing new anticancer drugs to enter the clinical arena in many years. This unique natural product with its novel mechanism of action and impressive clinical activity is a complex diterpenoid with a particular structure activity relationship. Taxol is under investigation as an antineoplastic agent in phase I, II and III human clinical trails. Target tumors include a variety of human cancers such as ovarian, breast, lung and colon as well as melanoma and lymphoma. Problems, such as limited supply of taxol and poor aqueous solubility and unsatisfactory stability have almost prevented taxol from advancing to clinical trials.

Progress has been made in increasing the supply, aqueous solubility, and stability without loss of its antitumor activity, (Hanson *et al.* 1994, Koepp *et al.* 1995, Rao *et al.* 1995), but as yet these new approaches have not clinically replaced the initial taxol:cremophor formulation which is a lipid vehicle and has been observed to cause life-threating anaphylactoid reactions in animals and humans (Koller and Dorr, 1994). Clinical application of taxol has often been limited by its side effects which include hypersensitivity reactions related to the rate and route of administration (iv) of its formulation (Gotaskie *et al.* 1994, and Koeller and Dorr, 1994). It has been reported that this kind of hypersensitivity reaction is associated with all drugs formulated in a cremophor formulation. Changing the route of administration and eliminating of cremophor from their formulations decreased or eliminated the incidence of hypersensitivity reactions (Howric *et al.* 1985 and Koeller and Dorr, 1994). This has led to an increased interest in developing the new approaches to formulation and administration of taxol and in heightening the need to understand the basic physicochemical properties of taxol. Hence, the oral route of administration was selected as an alternative to the iv route in order to reduce the dose-limiting side effects of taxol. Bulky structural drugs such as taxol usually exhibit low absorption rates which is a limiting factor in bioavailability. However, by the oral route a wide variety of formulation components are available enabling possible substitution. In addition, the oral route is the most convenient and the most widely used.

During the process of drug development, the oral bioavailability of a compound is a critical factor for the future of that compound as a drug. Different results (0 to 0.45%) have been reported for the oral bioavailability of taxol but from these data it is evident that the oral absorption of taxol is poor (Fujita H. *et al.*,1994, Sparreboom *et al.*, 1996). Low oral bioavailability has the consequences of more variable and poorly-controlled plasma concentrations and drug effect. Oral bioavailability is primarily controlled by the processes of drug solubility, dissolution, stability and absorption across the intestinal epithelium as well as the first pass clearance effect.

After oral administration of taxol as a suspension (10 mg/kg) or a solution of taxol in cremophor (10 mg/kg) and iv administration of a cremophor formulation to adult male Sprague-Dawley rats, taxol was not detected in the plasma of rats after oral administrations of taxol. However, comparison of area under the concentration versus time curve (AUC) between iv and oral (40 mg/kg) administrations using tritiated taxol a poor bioavailability (0.16%) for oral taxol was found.

Figure 1-8 show the possible fate of a drug administered orally in a liquid dosage form. In order to be effective: 1) the drug must be formulated to produce a suitable concentration in the GI tract, 2) the drug must be absorbed without substantial degradation and elimination in or through the GI tract. This work was aimed at accomplishing these objectives.

12.2 Physicochemical Parameters Responsible for Poor Oral Bioavailability of Taxol

To identify the mechanism(s) involved in poor bioavailability of taxol, preformulation studies of taxol, including pH-solubility profile, pH-stability profile, *in vitro* dissolution kinetics and *in vitro* intestinal absorption were conducted using phase solubility analysis, accelerated stability test, dispersed amount method, and the everted rat intestine technique, respectively. The aqueous solubility of taxol at 25° C is low (0.32 μg/ml) and increases linearly with temperature reaching (0.92 μg/ml) at 37° C in water. pH had an insignificant effect on the solubility of taxol (pH 1.2 - 8, $p < 0.05$). Taxol contains no ionizable groups in an acceptable pH range and therefore, pH adjustment did not produce a significant increase in solubility of taxol. Solubility of taxol in simulated stomach and intestinal fluids at 37° C is 0.83 and 0.98 µg/ml, respectively, which were close to that of water. However, solubility of taxol is sensitive to ionic strength in cremophor formulation (especially after addition of high concentration of salt), possibly due to salting out phenomenon.

Dissolution studies of taxol in water revealed that taxol is dissolved very rapidly but its solubility is so low that maximum saturable solubility reached quickly and dissolution of taxol is limited by its low solubility. Variation of pH (1.2-8.0) did not have a significant effect on dissolution rate of taxol. These data suggest that any improvement in solubility may enhance the dissolution rate of taxol.

The rate of degradation of taxol in water proceeded in a log-linear fashion and increased progressively with elevation of temperature. The pH-stability profile indicated rapid degradation of taxol at pH 1.2 and 8, with degradation $t_{½}$ of 1.6 and 1.8 days, respectively, at 37° C. At pH 7.4, the $t_{½}$ was 11.3 days. Maximum stability at 37° C was reached at pH 4.5 with $t_{½}$ of 26.9 days. Although taxol undergoes decomposition in aqueous media, after 8 hr incubation of taxol in simulated stomach and intestinal fluids at 37° C, more than 90% of the drug was found intact.

To identify the degree of contribution of low intestinal permeability to the poor bioavailability of taxol, the effect of dose (1-60 µg/ml) and formulation (suspension of taxol and solutions of taxol in cremophor EL) on gastrointestinal and region-specific absorption of taxol was investigated using rat everted intestine segments (ileum, jejunum and colon). Taxol was not detected in the serosal site of the intestine from the suspension formulation. The extent of absorption of a cremophor EL formulation by the everted intestine was poor (2% after 4 hour incubation) at 10 µg/ml concentration. However, increasing the soluble taxol concentration in the mucosal site from 10 to 60 µg/ml lead to an increase in the concentration of taxol in the serosal site of the intestine from 0.5 µg/ml to 1.6 µg/ml an indication of dose-dependency of absorption of taxol. No significant region-dependent variations in the intestinal absorption of taxol were detected. This study shows that the rate of taxol absorption is low and concentration-dependent.

. The taxol *n*-octanol:water partition coefficient has been reported to be more than 99 (Koller and Dorr, 1994), illustrating that taxol is a lipophilic compound and tends to interact with the cell membrane. In fact the results of permeability study of taxol through the immobilized artificial membrane column containing phosphatidylcholine (PC) analogues which are models of cell membranes, provide some evidence for that interaction. Hydrophobic-hydrophilic contribution of the drug's partitioning can be determined by IAM column because those compounds which do not have drug-membrane interaction will be eluated from the column fast. Retention time of taxol in IAM column in the presence of 0% acetonitrile was calculated to be 24 hr. Therefore, it seems that because of high lipophilicity taxol is well retained in IAM column and interacts with the membrane. For many drugs this high intraction is an indication of good absorption but in case of taxol everted rat gut and bioavailability studies were shown that taxol has poor absorptivity. As it was explained in chapter 9, although partitioning is normally the major contributor to drug permeation, passive permeation of the drug through cell membrane also depends on membrane diffusion coefficient of the drug (D_m). D_m significantly depends on the molecular size or molecular shape of drug. As molecular weight increases D_m dramatically decreases (Pidgeon *et al.* 1995). Therefore, in case of taxol, although it shows very good lipophilicity, it seems that the molecular weight dependency of drug absorption has a major contribution to the low oral bioavailability of taxol.

To summarize the first part of the project, it was found that taxol absorption appears to be dissolution rate-limited as well as permeation rate-limited. The observed low aqueous solubility, limited dissolution rate, limited gut permeability and chemical instability at pH 1.2 and 8 account for the low oral bioavailability of taxol.

12.3 Improving Oral Bioavailability of Taxol

The absorption rate and extent of a drug administered orally is controlled by many factors, among which the solubility and dissolution rate are the most important parameters. For a compound such as taxol, with limited dissolution due to its solubility, any improvement in solubility may enhance the dissolution of the compound. To improve the solubility of taxol through the utilization of clinically established agents, such as bile salts, cyclodextrins, nicotinamide, polyethylene glycol block copolymers and liposomes were investigated.

Among the cyclodextrins little or no effect was observed in αCD and βCD, but γCD and HPβCD showed a significant solubilizing effect. It appears that only some lipophilic part of taxol can fit into the lipophilic part of cyclodextrin, not the entire molecule. Therefore, HPβCD with a large cavity and high lipophilicity inside and high hydrophilicity outside the cavity had the largest solubilizing effect on taxol. Solubility increased linearly with HPβCD concentration to 50-fold, at 20 %w/v HPβCD, but nonlinearly in Nicotinamide, and bile salt solutions to 95-fold, and 40-fold, respectively. The solubility of taxol in liposome systems increased linearly in a lipid/taxol ratio-dependent fashion. Also in PEG-PBLA system concentration of up to 120 μg/ml of taxol was achieved. In comparison, the solubility of taxol achieved in 10 mg/ml liposomes concentration (DPPC:CHOL 2:1) was 96 μg/ml which was an indication of more than 96% association of taxol in liposome formulation while the solubility of taxol in 2 mg/ml PEG-PBLA concentration was an indication of only 40% association of taxol in PEG-PBLA system.

These studies showed that although the incorporation of taxol in liposomes and PEG-PBLA systems were effective methods for increasing the solubility of taxol, optimization of the experiment condition is not a simple task and many parameters need to be considered to increase the reproducibility and efficacy of these delivery systems. A disadvantage of a liposome system is its susceptibility to decomposition through hydrolysis and/or enzymatic action via bacteria *in vitro* and in the GI tract with subsequent leakage of entrapped solute. In addition, aggregation, particle size growth and leakage of encapsulated drugs may proceed at significant rates in aqueous suspensions of liposomes. Regular liposome formulations are unstable in the gut because of the presence of low pH and the enzyme, pepsin, in the stomach, detergent action of bile salts and the hydrolytic effects of phospholipases and other degrading enzymes such as trypsin, amino and carboxypeptidases in the gastrointestinal tract (Lasic and Papahadjopoulos, 1995). Therefore, in oral delivery of liposome formulation these obstacles should be considered. A disadvantage of the PEG-PBLA system may be their availability and cost and potential toxicities.

Dissolution studies of taxol in water as a function of nicotinamide, bile salts, HPβCD, and γCD concentration showed that the concentration of taxol, at any time, is substantially greater than that in pure water. This parallels the order observed for the equilibrium solubility of taxol in water and in the presence of different concentration of those solubilizing agents (Chapter 2). The dissolution rates (dc/dt) in these media increased as concentration of solubilizing agents increased (Chapter 4). Calculation of the relative dissolution rates of taxol in the nicotinamide, bile salts, HPβCD, and γCD medium (20%w/v) at the different time-intervals showed that at time 2 hr the relative dissolution rate of taxol in those solutions were 40-, 35- 22- and 18-fold higher than in water, respectively. However, Calculation of the $t_{50\%}$ of dissolution of taxol in the nicotinamide, HPβCD, and γCD medium showed that the $t_{50\%}$ of taxol in water increases as concentration of nicotinamide increases. The rapid dissolution of taxol ($t_{50\%} < 0.3$ hr) in the presence of low concentration of those solubilizing agents showed that taxol is dissolved very rapidly but its solubility is so low that maximum saturable solubility reached quickly and rate limiting step for poor availability is the poor solubility and not dissolution characteristics of taxol. Therefore any improvement in solubility of taxol can increased the dissolution rate of taxol. it was concluded that dissolution properties of taxol are crucial in its oral bioavailability and the rate and extent of oral absorption of taxol may be improved by using solubilizing agents.

All examined solubilizing agents had a significant effect on stability of taxol. HPβCD was particularly effective in retaining the stability of taxol followed by PEG-

PBLA block copolymer, liposomes, PEG 400, and nicotinamide in rank order. Stability of taxol dramatically decreased in bile salts solutions.

An interesting consequences of solubilizing taxol in these systems was the effects on the cytotoxicity of taxol against KB cell line. Our results shows that the cytotoxicity of HPβCD and nicotinamide, alone were lower than bile salts, and cremophor EL. The cytotoxicity of taxol in the presence of low concentrations (10^{-3} to 10^{-4} %) of HPβCD did not change, while decreased with nicotinamide and bile salts and especially increased with cremophor EL. However, in the presence of high concentrations (1.25% w/v) of solubilizers, bile salts, nicotinamide and HPβCD increased the cytotoxicity of taxol. These results show that it would be wise to consider the potential cytotoxicity effects of solubilizing agents alone and in combination with taxol in development and evaluation of new formulations of taxol.

Due to the observed improvement in solubility, dissolution, and stability, HPβCD and nicotinamide considered for everted rat gut permeability and oral bioavailability studies as suitable vehicles for delivery of taxol.

In the everted rat gut study, the amount of taxol absorbed from formulation containing HPβCD or nicotinamide were 2.7- and 4-fold more than in cremophor EL formulation (2%), respectively, indicating a role of absorption enhancer by these agents (Chaplin *et al.* 1991, Hirst *et al.* 1993, Hovgaard *et al.* 1995, Matsubara *et al.* 1995, Shao *et al.* 1994). Oral bioavailability studies in the rat also revealed that HPβCD and nicotinamide formulations increased in bioavailability of 6 and 14.6 fold (compared to cremophor formulation) respectively, with an absolute bioavailability of 0.97% and 2.3% which were superior to the other formulations. However, those *in vitro* and *in vivo* absorption studies did not show the mechanism involved in absorption enhancement of HPβCD and nicotinamide. Chaplin *et al.* 1991, Hirst *et al.* 1993, Hovgaard *et al.* 1995, Matsubara *et al.* 1995, Shao *et al.* 1994 addressed that nicotinamide and HPβCD can significantly change the biological and physiological function of cell membrane as well as physicochemical parameters of drugs.

Some insight of the mechanisms of action of nicotinamide and HPβCD *in vivo* model obtained from the IAM column studies. The permeation, P_m, of a drug through artificial membranes is directly proportional to partitioning of a drug into fluid membrane and membrane diffusion coefficient of the drug. Theoretically any changes in partitioning of the drug or diffusibility of a drug directly change the permeability of that compound through cell membrane.

Taxol makes complexes with HPβCD and nicotinamide. Complexation of drug with cyclodextrin and nicotinamide usually produces a product which has different polarity and molecular weight compare to drug alone and therefore, one of the consequences of this complexation could be a change in diffusion and partitioning of a drug through membrane. However, the results of permeability studies using IAM column chromatography showed that the retention time of taxol in the presence or absence of HPβCD and nicotinamide in the applied concentration range are constant. Therefore, it was concluded that due to instability of complexes in column (dilution effect of the mobile phase), presence of HPβCD or nicotinamide can not change the partitioning of taxol through the cell membrane.

However, by applying ^{51}Cr-EDTA as a transport marker it was found that in rat HPßCD and nicotinamide solutions of taxol significantly increased the %^{51}Cr-EDTA excretion in urine (0-8 hr) compare to the control (no drug) and the cremophor formulation. Therefore, it was concluded that HPßCD and nicotinamide do increase bioavailability of taxol through a change in permeability of the tight junctions in the endothelium of gut wall.

The most apparent side-effects of NSAIDs applied in the lower intestine which is increased in intestinal permeability (Abramson *et al.*, 1989, Davis 1996), Thus, applying a NSAID (flurbiprofen) prior to the formulation of taxol in cremophore showed that the bioavailability of taxol significantly increased from 0.16% (no flurbiprofen) to 23% (10 mg/kg flurbiprofen) in a dose-dependent fashion. This results show that HPßCD (20% w/v) and nicotinamide (20% w/v) may enhance the bioavailability of taxol through a change in permeability of tight junctions.

12.4 Conclusions

This work has contributed to an understanding of the role of certain physicochemical parameters responsible in the observed poor bioavailability of taxol and provided new knowledge of formulation approaches that have the potential of yielding a successful oral delivery form of taxol. These results highlighted the rule of poor solubility and permeability of taxol in overall low bioavailability of this remarkable anticancer drug and the potential application of HPβCD and nicotinamide as enhancers of solubility and permeability of taxol. In addition, we showed the capacity of NSAIDs as permeation enhancer for taxol.

12.5 Future Work

Future studies should concentrate on:

1) Optimization of polymer micellar systems and liposomes for oral delivery of taxol

2) The effect of other NSAIDs as well as other permeation enhancers on bioavailability of taxol

3) Combination effect of cyclodextrins and nicotinamide with NSAIDs on bioavailability of taxol

4) Effect of these solubility, stability, and permeation enhancers on over all antineoplastic activity of taxol

5) Effect of oral delivery of taxol on decreasing the side effects and especially the incidence of hypersensitivity reactions of taxol

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